The presynaptic protein Mover buffers synaptic plasticity at the hippocampal mossy fiber synapse

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“All models are wrong, but some are useful.”

George Box

To Cássio Abranches Viotti

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ABSTRACT

The increase in the complexity of brains in evolution is accompanied by a surprisingly small number of new synaptic proteins, in particular when considering the remarkably wider range of behavioral responses a primate shows in comparison with a roundworm. However, a few vertebrate-specific synaptic proteins arose. These proteins may convey specialization and complexity to vertebrate nervous systems, for example by increasing vesicle reloading speed, and maintaining or eliminating a synapse. Vertebrate-specific proteins, together with more elaborate circuits, could bridge the gap between simple and complex behaviors. But intricate machineries lead to complicated maintenance and, as a result, malfunctions occur. One of these vertebrate-specific proteins, Synuclein, is involved in Parkinson's disease. Another one, called Mover, is strongly upregulated in schizophrenia.

Mover is a synaptic vesicle-attached phosphoprotein, regulated by activity, and binds the conserved Calmodulin and the vertebrate-specific active zone protein Bassoon. Mover is differentially expressed at subsets of synapses. Knockdown of Mover in the calyx of Held leads to an acceleration of vesicle reloading after synaptic depression and to an increased calcium sensitivity of release.

In this study, I have used a Mover knockout mouse line to investigate the role of Mover in the hippocampal mossy fiber to CA3 pyramidal cell synapse and Schaffer collateral to CA1 synapse through extra- and intracellular electrophysiological recordings. While Schaffer collateral synapses were unchanged by the knockout, the mossy fibers showed strongly increased facilitation. The effect of Mover knockout in facilitation was both calcium- and age-dependent, having a stronger effect at higher calcium concentrations and in younger animals. Increasing cAMP levels by forskolin potentiated equally both wildtype and knockout mossy fiber synapses, but occluded the increased facilitation observed in the knockout. Blockade of Kainate receptors also occluded most of the increased facilitation observed in the absence of Mover.

These discoveries suggest that a) Mover has distinct roles at different synapses; b) generally acts to dampen the extent of presynaptic events; c) acts as a brake that can be released during low activity. I suggest a model in which Mover inhibits the Kainate receptor/cAMP pathway, which explains the observed results and supports the proposed role of Mover dynamically buffering synaptic strength. The results presented here are discussed in light of a possible role of this vertebrate-specific protein in scenarios such as schizophrenia, epilepsy, superpriming, synaptic plasticity and memory formation.

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1 INTRODUCTION

It is not rare for people to remember their first kiss, maybe decades ago, but forget where they left their keys less than an hour before. This is part of the normal functioning of the brain. Not only memory, but also movement, vision, audition and many other features are brought about by copious neurons, communicating with each other inside our brains.

1.1 Neurons

Neurons are electrically excitable specialized cells that are responsible for the functions that are unique to the nervous system (Brodal, 2010). They are cells comprised of three parts: soma, dendrites and axon. The soma is the cell body where the nucleus is located. Originating from the soma, several processes emerge. Dendrites are the processes that typically represent the receiving end of the neuron. Often, information will arrive to this neuron via the dendrites. That is not always the case, though. Information can also arrive to a neuron through the soma, or even through the axon. The axon is a process that also emerges from the soma and which carries the information forward. The information in this scenario is in the form of an electrical stimulus.

The electrical stimulus can be a current that passively spreads along the axon, or it can actively be propagated along the axon, typically in an all-or-nothing fashion. The latter form is called an action potential. The depolarization caused by the electric current can lead the neuron to fire an action potential, if it reaches a certain threshold. This action potential can travel along the axons, which are meters long in some cases. The information that this electrical stimulus carries can then be transferred to another neuron via specialized

connections called synapses. Information transfer at the synapse can happen in two ways: electrically or chemically. Electrical synapses allow for a more direct and unfiltered flow of electrical current from one neuron to another. Chemical synapses, on the other hand, require and enable a much more complex way of communication.

1.2 The Synapse

Chemical synapses communicate primarily using chemical molecules called neurotransmitters – hence the name. These are typically packed in vesicles and are released in response to a change in electrical potential in the synapse, provided by the arrival of the electrical stimulus, typically an action potential. The neurotransmitters are released into a cleft and bind to receptors on the receiving side of the synapse, this way propagating the message. Hence, the synapse can be divided into two opposing terminals. The first one belongs to the neuron that is *a priori* carrying the message and will have its neurotransmitter-laden vesicles released. It is called the presynaptic terminal. The other terminal belongs to the neuron receiving the message, now in form of neurotransmitters, and is called the postsynaptic terminal. Between the pre- and postsynaptic terminals is the synaptic cleft (Figure I.1, but see also Figure I.6).

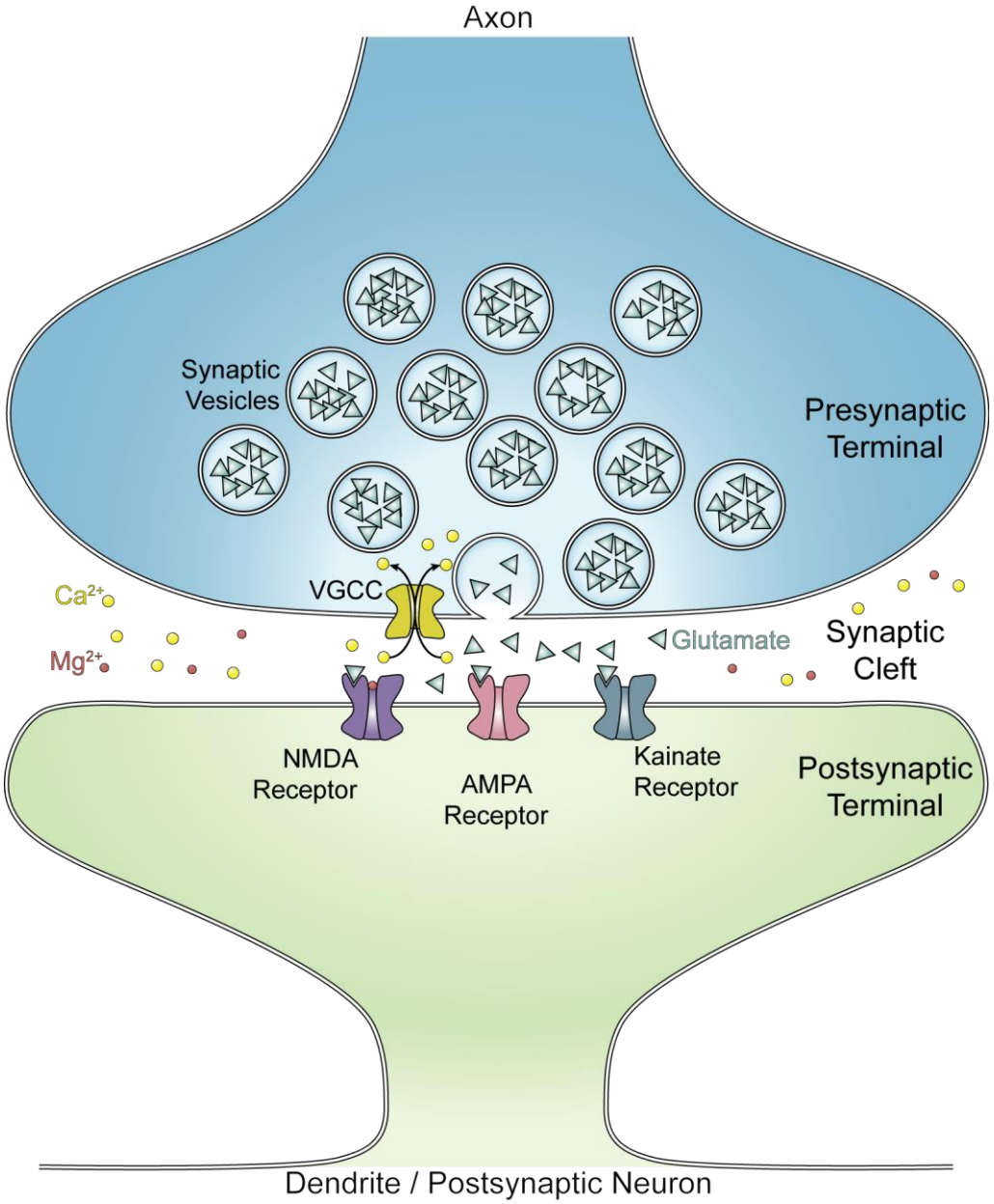

Figure I.1: Simplified representation of a synapse.
An action potential arrives through the axon to the presynaptic terminal (*blue*). The change in membrane potential leads to the opening of voltage-gated calcium channels (VGCC). The entry of Ca^{2+} triggers release of the neurotransmitters from the synaptic vesicles into the synaptic cleft. The neurotransmitter, in this case glutamate, binds to the receptors in the postsynaptic terminal (*green*) to transmit the signal to the postsynaptic neuron. The ionotropic receptors, when bound to the neurotransmitter, can open and let ionic currents through. In the case of the NMDA receptor Mg^{2+} blocks the channel pore at resting membrane potential, preventing the flow of ions unless the membrane depolarizes. For a more thorough description see the sections ahead.

1.2.1 The Postsynaptic Terminal

The postsynaptic terminal consists of what is typically understood as the receiving side of the synapse. Neurotransmitters released by the presynaptic terminal will bind to receptors in the postsynaptic membrane and carry the information to this neuron. It may seem counterintuitive to start the description with the terminal where neurotransmission ends, but bear with me. A lot of focus in this thesis will be given to the presynaptic terminal, and it will be easier to understand if one understands the postsynaptic terminal. For now, we only need to know that the presynaptic terminal releases neurotransmitters.

The postsynaptic terminal is typically part of a neuronal dendrite. They can be located in the dendritic shaft itself, or in protrusions called dendritic spines. However, the terminal can also be located in the cell body or even in an axon.

Attached to the postsynaptic membrane is an elaborate complex of proteins, called the post-synaptic density. This mesh of proteins contains scaffold and cytoskeletal components as well as receptors and proteins involved in metabolic cascades, such as kinases and phosphatases. Disturbances in proteins in the density have been linked to neurological and psychiatric diseases (e.g. Bayés et al. 2011), emphasizing the importance of the integrity of the post-synaptic density. For a more thorough view on the postsynaptic density see Sheng & Kim 2011. Proteins in the density are also involved in anchoring and trafficking of neurotransmitter receptors. Let us take a deeper look at these receptors.

The Neurotransmitter Receptors

Arguably, one of the most important groups of proteins in the postsynaptic terminal are the neurotransmitter receptors. They are typically membrane proteins that are activated when bound to a specific neurotransmitter. Receptors can, therefore, be categorized in terms of their response to specific neurotransmitters. There are glutamate receptors, gamma-aminobutyric acid (GABA) receptors, acetylcholine receptors, glycine receptors, and many others.

Of special interest to this thesis are the glutamate receptors. These can be separated again in different families: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, kainate receptors, N-methyl-D-aspartate (NMDA) receptors and metabotropic glutamate receptors (mGluRs), which will be mentioned again later.

Receptors can also be divided in terms of how they transduce the signal. There are two categories: ionotropic and metabotropic receptors. Ionotropic receptors contain an ion channel in their transmembrane domain. Metabotropic receptors, on the other hand, do not contain ion channels in their structure but act through activation of G-proteins.

Metabotropic receptors have an extracellular domain, which binds the neurotransmitter, and an intracellular domain, which binds to G-proteins. Because of this interaction they are also called G-protein-coupled receptors. The G-proteins, after uncoupling from the receptor upon ligand-binding, can either directly activate ion channels or lead to a cascade of metabolic events. This cascade of events can amplify the initial signal and can also lead to opening or closing of ion channels.

Ionotropic receptors, however, are ion channels themselves. They have an extracellular domain to which the neurotransmitter binds, and a membrane-spanning domain that forms the channel. When the specific agonist (molecule that binds to a receptor and activates it) binds to the ionotropic receptor it leads to the opening of the channel, allowing specific ions to pass through.

Either directly (ionotropic) or indirectly (metabotropic), the activation of neurotransmitter receptors usually affects ion channels. We will now take a closer look at them, but if you want a more detailed view on the molecular machines at the postsynaptic terminal, refer to Kennedy (2000).

1.2.2 Ion Channels and ionic currents

I would like to take a short break on the synapse structure to explain a bit more on ion channels and their effect on neurotransmission. Ion channels are not, by any means, exclusive to the synapse, but will be explained here so we can better understand synaptic transmission. The study of ion channels is an incredibly vast topic that is pivotal for the understanding of neuroscience, as well as many other fields in biology. Hence, I will only brush on some basic ideas for us to better comprehend neurotransmission. For the scientist who wants to dive into electrophysiology and wants to better understand the role of ion channels in cellular signaling I would recommend the book Ion Channels of Excitable Membranes (Hille, 2001).

As mentioned before (see section ‘The Neurotransmitter Receptors’), channels can open and let ions flow through when a ligand (e.g. a neurotransmitter) binds to them. There are,

however, various other means and mechanisms by which channel activity can be regulated. Opening or closing of a channel can be brought about not only by ligands (such as neurotransmitters), but also by other factors, such as membrane potential (e.g. voltage-gated calcium channels) and light (e.g. channelrhodopsins). Ion channel states are also more complex than just 'open' or 'closed' – they can also be 'inactivated', which is another conformation of the 'closed' state. Additionally, the ion flow through the channels can be modulated: allosteric ligands can influence the channel state and ions can block the ion pore, just to mention a couple of examples.

One relevant example of a channel that can be blocked by ions is the NMDA receptor. At neuronal resting membrane potential (usually considered around -70mV), Mg^{2+} (or Zn^{2+}) blocks NMDA receptors so that, even if glutamate is bound to it, no ion flow will occur. For the Mg^{2+} ion to leave the channel, a depolarizing event has to take place. NMDA receptors are therefore also considered coincidence detectors: two conditions need to coincide for the channel to open – binding of glutamate and depolarization of the membrane.

When open, different channels have different permeability to ions leading to different results. Voltage-gated calcium channels (VGCCs), for example, as the name suggests, are permeable to Ca^{2+}, but also slightly permeable to Na^{+}. NMDA receptors are permeable to Ca^{2+}, Na^{+} and K^{+}. AMPA and Kainate receptors are generally more permeable to Na^{+} and K^{+}. However, their permeability depends on the subunit composition of the channel. Hence, these receptors can also permeate Ca^{2+} depending on the subunits present.

Calcium influx into the cytoplasm of a cell can trigger several different metabolic paths. The effect of calcium influx on neurotransmitter release, for example, will be described further ahead in 'Calcium-triggered vesicle release' (Section 1.2.3.1). Overall, one important consequence of ion flow through a channel is its effect on the membrane potential. The outcome of opening an ion channel will mostly depend on the charge of the ion and the direction and strength of their flow. The direction of their flow is governed by the electrochemical driving force the ions are subject to. The driving force will depend on the concentration of the ion inside and outside the cell, its charge and the membrane potential. From this, it is possible to make certain generalizations. When channels open at resting membrane conditions, Na^{+} and Ca^{2+} will usually enter the cell, while K^{+} and Cl^{-} will usually leave the cell. However, as this depends on the ionic driving forces, there can be exceptions.

It is important to understand is that the flow of ions through the channel will create an electric (ionic) current. The direction of the current is defined as the direction of the flow of positive charges. Therefore, an influx of positive ions (for example Na^+) into the cell creates an inward current. Conversely, an efflux of positive ions (for example K^+) out of the cell creates an outward current. A flow of negative ions (for example Cl^-) into the cell also results in an outward current, as per definition the positive charges will flow out of the cell.

Electric currents entering or leaving the neuron will have an impact on that neuron's membrane potential. Inward currents tend to depolarize the membrane. If this depolarization crosses a certain threshold, the neuron can fire an action potential and carry on that information to other neurons. Neurotransmitters or effects that lead to membrane depolarization are therefore known as excitatory. By extension, outward currents will lead to hyperpolarization and can prevent a cell from firing action potentials. Because of this, neurotransmitters that lead to hyperpolarization are known as inhibitory.

When a neurotransmitter binds to a receptor in the postsynaptic neuron, it can create a current through the membrane of this cell. This is referred to as a postsynaptic current (PSC). If this current is the result of the binding of an excitatory neurotransmitter, such as glutamate, the current is termed an excitatory postsynaptic current (EPSC). If it comes from an inhibitory neurotransmitter, such as GABA, it is usually an inhibitory postsynaptic current (IPSC). As described before, the currents can lead to changes in membrane potential. We, therefore, have also excitatory and inhibitory potentials (EPSP and IPSP, respectively).

As mentioned before, the presence of ion channels is not exclusive to the postsynaptic terminal. It is not even exclusive to synapses or neurons. And even though the synaptic receptors, which let current flow in or out of the synapse, are vital for synaptic transmission, they are not exclusive to the postsynaptic terminal. The flow of information is not always so unidirectional, i.e. from pre- to postsynaptic terminals. There are forms of neurotransmission that do not follow this classical pathway. Neurotransmission in the cannabinoid system, for example, has been shown to act from post- to presynaptic terminal, in a kind of retrograde signal (Ohno-Shosaku et al., 2001). Nitric oxide can also act as a neurotransmitter and does not follow the typical direction of signaling (Snyder, 1992). It becomes clear, therefore, that there are also receptors in the presynaptic terminals.

Hence, we will now take a closer look at what happens in this neurotransmitter-releasing side of the synapse.

1.2.3 The Presynaptic Terminal (and Neurotransmitter Release)

The presynaptic terminal is an incredibly specialized structure. Its main function is to release neurotransmitter-laden synaptic vesicles in a precise and controlled manner. For that, it relies on a complex machinery, which includes several protein families like SNAREs (Soluble N-ethylmaleimide sensitive factor Attachment protein REceptor), SM proteins (Sec1/Munc18-like proteins), Rab proteins and others. Responsible for the fusion of the vesicles with the plasma membrane are the SNARE proteins (see Jahn & Scheller 2006 for a review). SNARE proteins contain a domain formed by heptad repeats that form coiled-coil structures. The core SNARE proteins, necessary for synaptic vesicle fusion, are Vesicle-Associated Membrane Protein (VAMP, also known as Synaptobrevin; Trimble et al. 1988), Syntaxin (Bennett et al., 1992) and SNAP25 (synaptosomal-associated protein of 25kDa; Oyler et al. 1989). VAMP, present in the vesicular membrane, forms a complex with Syntaxin and SNAP25, which are attached to the plasma membrane (Söllner et al., 1993a, 1993b). This is called the SNARE complex.

The formation of this complex brings the plasma membrane and the vesicular membrane into close proximity and leads to the formation of the fusion pore. The membrane of the vesicle opens and fuses with the plasma membrane, and a connection between its lumen and the synaptic cleft is established. The neurotransmitter molecules can now flow out of the vesicle into the synaptic cleft (for more on this see Rizo & Rosenmund 2008 or Südhof & Rothman 2009). SNARE proteins, however, do not work alone: other proteins participate to assure high efficiency and accurate timing of neurotransmitter release. The protein Munc18, for example, is part of the conserved core fusion machinery and has been proposed to start the process of fusion through its binding to Syntaxin-1 (Ma et al., 2013). Munc13 in its turn is responsible for rendering the vesicles release-ready, in a process called vesicle priming (Varoqueaux et al., 2002). Deletion of either Munc18 or Munc13 leads to an arrest in neurotransmitter secretion (Verhage et al., 2000; Varoqueaux et al., 2002).

For a vesicle to become fusion-competent, as mentioned, it needs to be primed. Two different forms of priming are usually considered necessary for a synaptic vesicle to be releasable upon arrival of the action potential into the terminal: molecular and positional

priming (for an interesting discussion on the topic see Neher & Sakaba 2008). Molecular priming corresponds to the buildup of the release apparatus, while positional priming corresponds to the placement of the vesicle in the proximity of the calcium channels. The reason why this proximity is important will be discussed in the following sections 'Calcium-triggered vesicle release' and 'The Active Zone and Calcium Microdomains'.

It is also important to notice that the presynaptic terminal, like the postsynaptic side, contains neurotransmitter receptors. As described in the section about the postsynaptic terminal, the presynaptic terminal possesses receptors for nitric oxide and for endocannabinoids. In addition, receptors for the neurotransmitters that the synapse releases are also present in the presynapse. That is why they are referred to as autoreceptors. These receptors are often, but not always, responsible for feedback inhibition, inhibiting neurotransmitter release (e.g. Kamiya et al. 1996, Schmitz et al. 2001). As they are often modulating neurotransmitter release in a dynamic way, they will be further explained at the section on Synaptic Plasticity (Section 1.3).

1.2.3.1 Calcium-triggered vesicle release

The complex release machinery allows for exocytosis of neurotransmitters in a fast and precise manner. But how does the synapse know it is time to release neurotransmitters? What is the signal for the synaptic vesicles to fuse? The answer is calcium. An increase in calcium concentration inside the presynaptic terminal is sensed by Synaptotagmin (Syt), which is then the trigger for release (Perin et al., 1990). Synaptotagmins contains two cytoplasmic C2 domains, which mediate calcium dependent phospholipid binding (Brose et al., 1992). It was indeed found that Syt1 was essential for the precisely-timed calcium-triggered release, but not required for fusion itself (Geppert et al., 1994). Syt2 and Syt9 have also been described to serve as the calcium sensor for synchronous release in some synapses (Xu et al., 2007). Other proteins may serve as calcium sensors as well, but provide slower, different kinds, of release. For this study, two of these proteins are especially noteworthy. The first is Syt7, which has been proposed to be the calcium sensor required for synaptic facilitation (discussed ahead in 1.3 Synaptic Plasticity; Jackman et al. 2016). The other one is a protein, part of the cytomatrix of the active zone, called Piccolo. This is a vertebrate-specific protein and it has been proposed to act as a low-affinity calcium sensor for vesicle release (Gerber et al., 2001; Garcia et al., 2004).

Where does this rise in Ca^{2+} concentration come from? How does the calcium enter the presynaptic terminal? The answer is simple: calcium channels. More specifically voltage-gated calcium channels (VGCCs, for a review on different VGCCs and their discovery see Dolphin 2009). When the action potential invades the presynaptic terminal, the depolarization of the terminal can lead to the opening of the calcium channels. These channels are not randomly located in the synapse. They are tethered to the active zone through their binding to Rab3-interacting molecules (RIM; Kaeser et al. 2011). This leads us to the question: what is the active zone?

1.2.3.2 The Active Zone and Calcium Microdomains

The active zone is where the synaptic vesicles are released (for a review see Südhof 2012). This may sound simple, but there is a whole plethora of proteins that come together to assemble an active zone. This electron-dense mesh of proteins is called the cytomatrix of the active zone, and includes the aforementioned proteins Munc13, Piccolo (also known as Aczonin) and RIM, but also RIM-binding proteins (RIM-BPs), ELKS (also known as CAST), Liprins-α, and Bassoon. In addition, the precise localization is important, as the active zone has to assemble exactly opposite to the postsynaptic density.

Organization of the active zone allows for calcium channels, vesicles and the calcium sensor to be close together (Ackermann et al., 2015). Such proximity allows for an increase of calcium in so-called microdomains (Chad and Eckert, 1984; Simon and Llinás, 1985; Neher, 1998). That means that the increase in calcium is much higher in these microdomains, i.e. in the proximity of the calcium channels, which can allow for the exocytosis of the release-ready vesicle without the entire terminal being flooded by calcium. In some synapses, the vesicles and the calcium channels are so closely associated that this is referred to as a nanodomain (Augustine et al., 2003; Bucurenciu et al., 2008; Eggermann et al., 2012). This evokes the idea that the closer the calcium sensor is to the calcium channel, the less calcium influx is needed for the vesicle to be released and that, by extension, a vesicle has a higher chance to be released if it is closer to the channel (coupling distance). This leads us to the idea of vesicular release probability.

The Release Probability

A readily releasable vesicle has a higher chance of being released when it is close to a calcium channel (e.g. Chen et al. 2015). Therefore, upon arrival of an action potential into

the presynaptic terminal, vesicles that can be released will be released with a certain probability. Not all vesicles are released upon opening of the calcium channels. Not even all primed and docked vesicles are released. Fusion-competent vesicles are subject to a vesicular release probability (P_{vr}).

I would like to take a moment here and approach an issue that often confounds discussions about release probability. This has confused me as a young electrophysiologist and has confused others as well. When referring to release probability it is useful to make a distinction that is not always explicit: the difference between synaptic release probability (here referred to as P_r) and vesicular release probability (P_{vr}). P_{vr} is the "probability with which a particular vesicle in the readily releasable pool (RRP) can be stimulated for exocytosis by Ca^{2+} influx during an action potential", whereas P_r is "the probability with which an action potential stimulates neurotransmitter release at a synapse" (Fernández-Chacón et al., 2001). This distinction can be trivial at times, but essential at others.

The interaction between P_r, P_{vr}, RRP and synaptic strength can be described through this equation (Neher, 2017):

$$PSC = N \times p_{occ} \times p_{succ} \times q$$

Where:

- *PSC* is the potsynaptic current resulting from the arrival of one action potential;
- *N* is the number of release sites;
- p_{occ} is the probability of these sites being occupied by release-ready vesicles;
- p_{succ} is the probability that an attached vesicle will actually be released upon the arrival of an action potential (it is, therefore, a synonym of what was defined here as P_{vr});
- q is the response to the release of one vesicle (quantal size).

Some other parameters can be derived from this equation. For example, the RRP size refers to the number of sites currently being occupied ($N \times p_{occ}$). Using this equation, P_r can be equated to the product between RRP and P_{vr} (hence: $N \times p_{occ} \times p_{succ}$). P_r is, therefore, a measure of synaptic reliability since it represents the likelihood that at least one vesicle will be released by the arrival of an action potential (Alabi and Tsien, 2012).

Since *N* is believed to be a fixed number (Vere-Jones, 1966; Neher, 2017), changes in P_r reflect a change in P_{vr}, p_{occ}, or both. It is important to notice that even though *N* is fixed, an

increase in the RRP size can be brought about by an increase in p_{occ}. Furthermore, some methods of estimation of release probability measure not P_r or P_{vr} but the product $p_{occ} \times p_{succ}$ (Scheuss and Neher, 2001; Neher, 2017). Also noteworthy is the fact that P_{vr} is heterogeneous across different vesicles, but, for the applications here, assuming a homogeneous P_{vr} is sufficient (Neher, 2015). Additionally, the dynamic changes that can happen in p_{occ} and p_{succ} during activity will addressed below in the section about Synaptic Plasticity.

As aforementioned, the proximity between vesicle (or better, the calcium sensor) and a calcium channel will influence P_{vr}. However, there are also other factors that influence P_{vr}, such as the strength of the calcium influx. A stronger influx will lead to a higher P_{vr}. This is clear since we know that calcium is the trigger for release: the more calcium, the more release. It is clear that the number of calcium channels plays an important role, as it correlates with the amount of calcium influx (Schneggenburger et al., 2012). Additionally, the single-channel current can influence the vesicle release and shape the amplitude of the microdomain. The presence or lack of calcium buffers, their on-rate, concentration and mobility additionally play a role (Schneggenburger et al., 2012; Delvendahl et al., 2015).

The "willingness" of the synaptic vesicle to be release also influences P_{vr}. This willingness can be interpreted as how tightly the release machinery is adjusted. The aforementioned priming factor Munc13 contains a C1 domain that binds diacylglycerol (Betz et al., 1998). The activation of this site has no effect on the priming activity of this protein, but it reduces the energy needed for fusion, therefore reducing P_{vr} (Basu et al., 2007). Another priming factor that has been show to behave similarly are the complexins (Schotten et al., 2015). Vesicular glutamate transporters (VGLUTs), the proteins responsible for filling the synaptic vesicles with the excitatory neurotransmitter glutamate, also influence the release probability. VGLUT1-containing neurons have been shown to have a lower P_{vr} than VGLUT2 and 3 due to VGLUT1's ability to bind and inhibit endophilin A1, a positive regulator of exocytosis (Weston et al., 2011). Not only which isoform is present, but also the number of copies influences release: a reduction in the expression of VGLUT translates into reduced P_{vr} (Herman et al., 2014). Another synaptic vesicle protein regulates P_{vr}: Mover (Körber et al., 2015). This protein is the main topic of this thesis and, therefore, it deserves a separate section for itself in the introduction. Thus, for more on Mover see section 1.5. Since these proteins usually change the amount of Ca^{2+} that is necessary for exocytosis it is said they affect the calcium sensitivity of release.

One interesting protein that binds to Munc13 but influences P_{vr} through a different mechanism (activation of Calmodulin-dependent Kinase II) is Calmodulin (Pang et al., 2010). Since it also binds to Mover (Körber et al., 2015) it will be described in more detail in section 1.5.2.

Other proteins have been implicated in influencing P_{vr} [e.g. Rab3s (Schlüter et al., 2004, 2006); G-protein-coupled Receptor Kinase-interacting Protein 1 (GIT1) (Montesinos et al., 2015)]. Therefore, for more details on how proteins affect release probability see Körber & Kuner 2016.

Superpriming

As mentioned above, many different factors influence release probability. One of these effects is of particular interest, as Mover is proposed to be involved in this process, is superpriming (Körber and Kuner, 2016). It is a process proposed to happen to a subset of synaptic vesicles, belonging to a fast-releasing pool of vesicles (Taschenberger et al., 2016).

The proposed effect of superpriming is that it would grant a higher P_{vr} to some readily-releasable vesicles (Schlüter et al., 2006; Lee et al., 2013; Taschenberger et al., 2016). There is some controversy as to which mechanism leads to this higher P_{vr} in these superprimed vesicles. While Ishiyama et al. (2014) propose that the change in P_{vr} could be explained by a change in vesicle-channel proximity (positional priming), most evidences argue that superpriming is the result of a maturation of the vesicle's intrinsic Ca^{2+} sensitivity (molecular priming) due to conformational changes in Munc13 (Basu et al., 2007; Lee et al., 2013; Taschenberger et al., 2016; Michelassi et al., 2017).

Furthermore, superpriming is a slow process: in the calyx of Held it has a time constant of around 4 seconds, whereas mobilization of reluctant vesicles into the fast-releasing pool has a time constant of 60 ms (Lee et al., 2013). Thus, it mostly affects synaptic strength during sparse activity or the initial response to a burst of activity. Additionally, it is proposed that the superpriming of a different fraction of synaptic vesicles could explain the variability in the calyx of Held short-term plasticity (Taschenberger et al., 2016). Hence, let us understand what synaptic plasticity is.

1.3 Synaptic Plasticity

All this complicated machinery that brings about synaptic transmission only makes sense if we think that, between a neuron and another, the information needs to be processed in some way. This is not a simple, passive, diffusion of a signal from one neuron to another. As described before, the electrical signal is transformed into a chemical signal, just to be transformed into electrical signal again in the following neuron. All that happens via a very precise and well-regulated machinery of release of synaptic vesicles and regulated receptors. That means that the synapse cannot be just a simple layover, where information gets transmitted from one point to the other exactly as it was. On the contrary, synapses compute, they analyze, they can be considered the basic unit of computation in the brain (Zador, 2000).

Even though they are often interconnected, it is possible to differentiate two types of synaptic plasticity: structural and functional.

Synapses can assemble and disassemble, grow and shrink. Morphological changes like that are described as structural plasticity. Dendritic spines (first mentioned in section 1.2.1 'The Postsynaptic Terminal'), for example, show substantial change in morphology in response to neuronal activity. Induction of strengthening of the synapse leads to engorgement of the spine, while a reduction of transmission efficacy leads to shrinkage. For a review on spine dendritic plasticity see Bosch & Hayashi 2012.

Memories are believed to be stored in form of synapses. These could be the result of newly formed synapses or strengthening of former synapses (e.g. Hill & Zito, 2013; Liu et al., 2013). Since formation of new synapses can be interpreted as structural plasticity and strengthening of old synapses is functional plasticity, it becomes clear that plasticity is vital in learning and memory. More on memory and cognition will be discussed in the section about the hippocampus (1.4 'The Hippocampus'), but if you are already thirsty for a deeper understanding of what is memory check Poo et al. (2016).

Let us understand in more detail what functional plasticity is. It can be described as a change in synaptic transmission efficacy and it can vary enormously when comparing different synapses. This change in efficacy can be an increase or a decrease in the resulting current in response to an action potential. It can be a result of changes in the presence of postsynaptic receptors (i.e. q [refer to equation on page 21]), in the presence of synaptic

vesicles (i.e. RRP), the states in which the channels can be found, the amount of influx of calcium into a terminal, the amount of calcium already present at the terminal, and other presynaptic factors that can influence P_{vr}. Importantly, it can also vary in the terms of it being short- or long-lasting.

1.3.1 Short-Term Plasticity

When two action potentials arrive in a presynaptic terminal within a short interval, they will usually trigger different responses. This is a typical example of short-term plasticity (STP). It refers to these changes in synaptic transmission in the scale from milliseconds to minutes.

Contrary to what an inexperienced neuroscientist might expect, short-term plasticity is usually the rule, not the exception, in synaptic transmission: possibly all synapses are regulated by brief transient processes leading to strengthening or weakening of the responses. It is easy to understand this when one realizes that it is through plasticity that a lot of the computation of the brain is done (Abbott and Regehr, 2004). In terms of filtering, synapses with a low initial release probability tend to act as high-pass filters, whereas synapses with high probability act as low-pass filters. These ideas will be elaborated upon below, but the importance of the release probability already hints to the importance of presynaptic processes in short-term plasticity.

Before going deeper into the mechanisms that bring about plasticity, the proper nomenclature should be introduced. A decrease in synaptic strength is referred to as synaptic depression. For example, an action potential elicits an EPSC of 120 pA. A second action potential then arrives at the synapse within a short time frame, and evokes an EPCS of 80 pA (Figure I.2A). On the other hand, if the second action potential had evoked a response of, for example, 300 pA, the synapse would have undergone facilitation. Facilitation is, therefore, a form of synaptic enhancement: an increase in the synaptic strength (Figure I.2C).

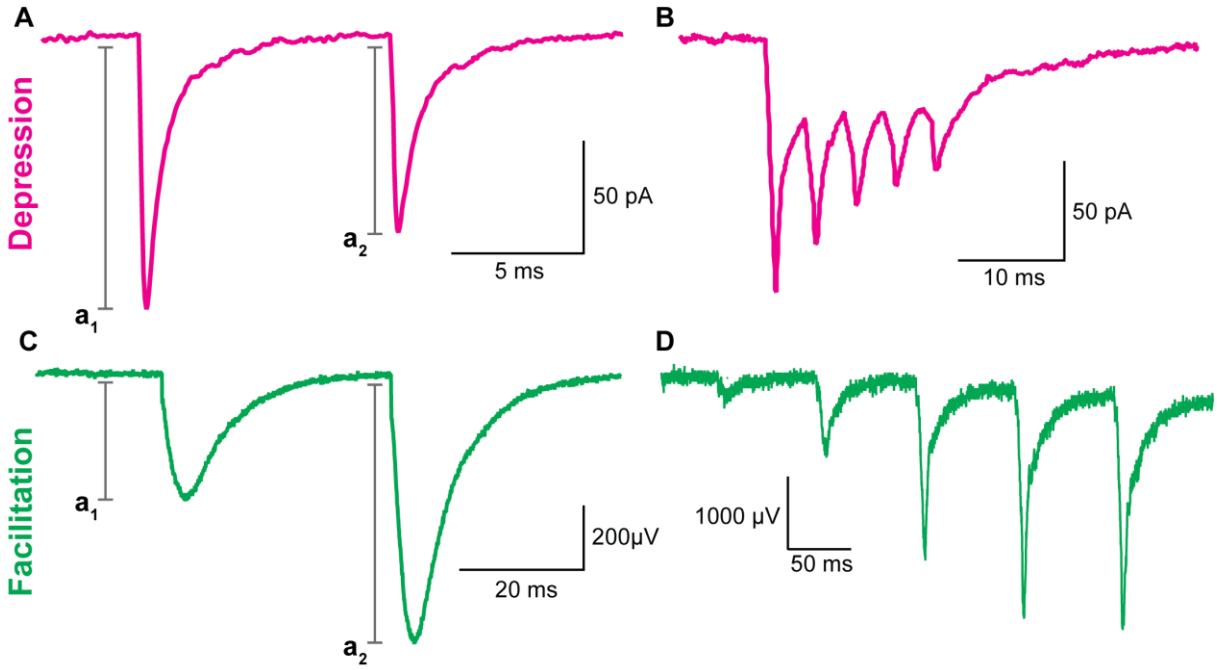

Figure I.2: Examples of short-term synaptic plasticity: facilitation and depression.
Recordings of current (*A, B*) or voltage (*C, D*) traces representing synaptic facilitation (*green*) and depression (*magenta*). Paired-pulse ratio (see text) is defined as the division of the amplitude a_2 by a_1. (*A, B*) Personal recordings (modified from Viotti, 2013) from cerebellar mossy fiber evoked EPSC illustrating paired-pulse (*A*) and a 300 Hz train of 5 stimuli (*B*). (*C*) Personal recording (unpublished) from Schaffer collaterals field EPSP illustrating paired-pulse facilitation. (*D*) Personal recording (unpublished) from hippocampal mossy fiber field EPSP illustrating facilitation in a 25 Hz train of 5 stimuli.

1.3.1.1 Synaptic Facilitation

There is often a distinction between different forms of short-term synaptic enhancement. Besides facilitation, augmentation and post-tetanic potentiation are other forms of enhancement. They differ in their kinetics. Since augmentation and post-tetanic potentiation are closely related forms of plasticity usually evoked by rather long trains of action potentials and this is something which has not been done in this thesis, a focus will be given to facilitation. For more information on these forms of synaptic enhancement see Fioravante & Regehr 2011.

While facilitation is evoked by repeatedly stimulating neurons, there are slightly different forms of doing that. If pairs of stimuli are used, that is two stimuli given within a short time interval, this is termed paired-pulse facilitation. The measurable increase in response is also called paired-pulse facilitation, or paired-pulse ratio (since it can also be used to denote depression). It is calculated as the second response - usually its amplitude, charge or slope - divided by the first response (Figure I.2A and C). Responses in this scenario are

often EPSPs or EPSCs (discussed in section 1.2.2 ‘Ion Channels and ionic currents’). Since the parameter usually measured is the amplitude of these potentials or currents, the amount of response will be henceforth referred to as its amplitude. The stimulation using more than two stimuli is called a train of stimuli (Figure I.2B and D). The facilitation there is also measured as the ratio between the amplitude in response to stimulus n and the amplitude in response to the first stimulus.

Facilitation usually has a time scale of tens to hundreds of milliseconds (Zucker and Regehr, 2002). There are, however, exceptions. Facilitation with a much longer time scale is seen at the hippocampal mossy fiber synapses (Salin et al., 1996). In this synapse, low-frequency stimulation (e.g. 0.1 Hz) successfully elicits facilitation. This means that this is a form of facilitation that decays with a time course of more than 10 seconds (see also section 1.4.1 The Mossy Fibers).

Proposed Mechanisms of Facilitation

What brings about facilitation? Which mechanisms allow for a short-term enhancement of synaptic activity? Postsynaptic processes rarely contribute to facilitation. One postsynaptic mechanism that can contribute to facilitation is the release by depolarization of polyamine blocks of AMPA receptors, which leads to an activity-dependent increase in postsynaptic currents (Rozov et al., 1998; Rozov and Burnashev, 1999). However, postsynaptic involvement appears infrequent: most proposed mechanisms for facilitation involve an increase in the number of vesicles released.

The most prominent proposed mechanisms of facilitation involve interplay between calcium signaling and vesicle exocytosis. The invasion of a presynaptic terminal by an action potential leads to an increase of calcium concentration to tens or hundreds of micromolar in the proximity of the calcium channels (Simon and Llinás, 1985; Yamada and Zucker, 1992). However, the concentration drops steeply with distance from an open channel because of the diffusion of the ions and their capture by calcium buffers (Neher, 1998). This brief and spatially constrained calcium concentration is therefore called ‘local calcium’ and can be sufficient to activate fast, low-affinity sensors (such as Syt1) in the vicinity. The rapid aspect of the calcium signal and the kinetics of Syt1 allow for a fast and synchronous release. But, since calcium diffuses and binds to buffers, a much longer but weaker calcium signal persists in the terminal for hundreds of milliseconds: the ‘residual calcium’ (Figure I.3A).

The residual calcium could lead to a summation of the calcium signal and, therefore, stronger vesicle release. This could help explain synaptic facilitation. However, in many synapses the residual calcium alone is too weak (hundreds of nanomolar) to explain a significant increase in the calcium signal when summed with the local calcium (hundreds of micromolar; Felmy et al. 2003). As a result, it has been proposed that calcium is acting at a site that is different from the aforementioned fast, low-affinity site triggering secretion, like in Syt1 (Figure I.3B; Zucker & Regehr 2002; Fioravante & Regehr 2011). Recently, Syt7 has been shown to fulfill the necessary characteristics for being a calcium sensor for facilitation. It has a high calcium affinity and slow kinetics that make it a good candidate for this role and indeed, when knocked out, facilitation was mostly gone in all synapses tested (Jackman et al., 2016).

It was mentioned that facilitation was "mostly gone" in the absence of Syt7, which means there was still some degree of facilitation left, notably in the hippocampal mossy fiber synapses (Jackman et al. 2016; for more on this synapse see section 1.4.1 'The Mossy Fibers'). A proposed mechanism that contributes to facilitation at these synapses is spike broadening (Figure I.3C; Geiger & Jonas 2000). The inactivation of K^+ channels during high-frequency stimulation leads to a prolonged action potential waveform. This prolonged waveform leads to a longer calcium influx and, consequently, to stronger neurotransmitter release. This is, therefore, a use-dependent increase in calcium influx.

A fourth proposed mechanism for synaptic facilitation is that of endogenous buffer saturation (Figure I.3E). The idea here is that calcium-binding proteins usually capture some of the calcium ions before they trigger release, reducing the release probability. A second wave of calcium entry would face, therefore, less buffers in its way to promoting vesicle secretion and would lead to facilitation. This mechanisms is also proposed to contribute to facilitation at the mossy fibers (Blatow et al., 2003; Vyleta and Jonas, 2014).

There is another proposed mechanism of use-dependent increase in calcium influx: calcium-dependent facilitation of calcium entry (Figure I.3D). Contrary to the other proposed mechanisms, this one has not been observed in the mossy fiber synapse. In this mechanism the entry of calcium works as a positive feedback and leads to an increase in calcium currents. One important calcium sensor associated in calcium channel modulation is CaM, which can bind to the carboxy tail of some calcium channels leading to facilitation (DeMaria et al., 2001). More information about CaM in the section 1.5.2. For a deeper discussion on the mechanisms responsible for facilitation see Jackman & Regehr 2017.

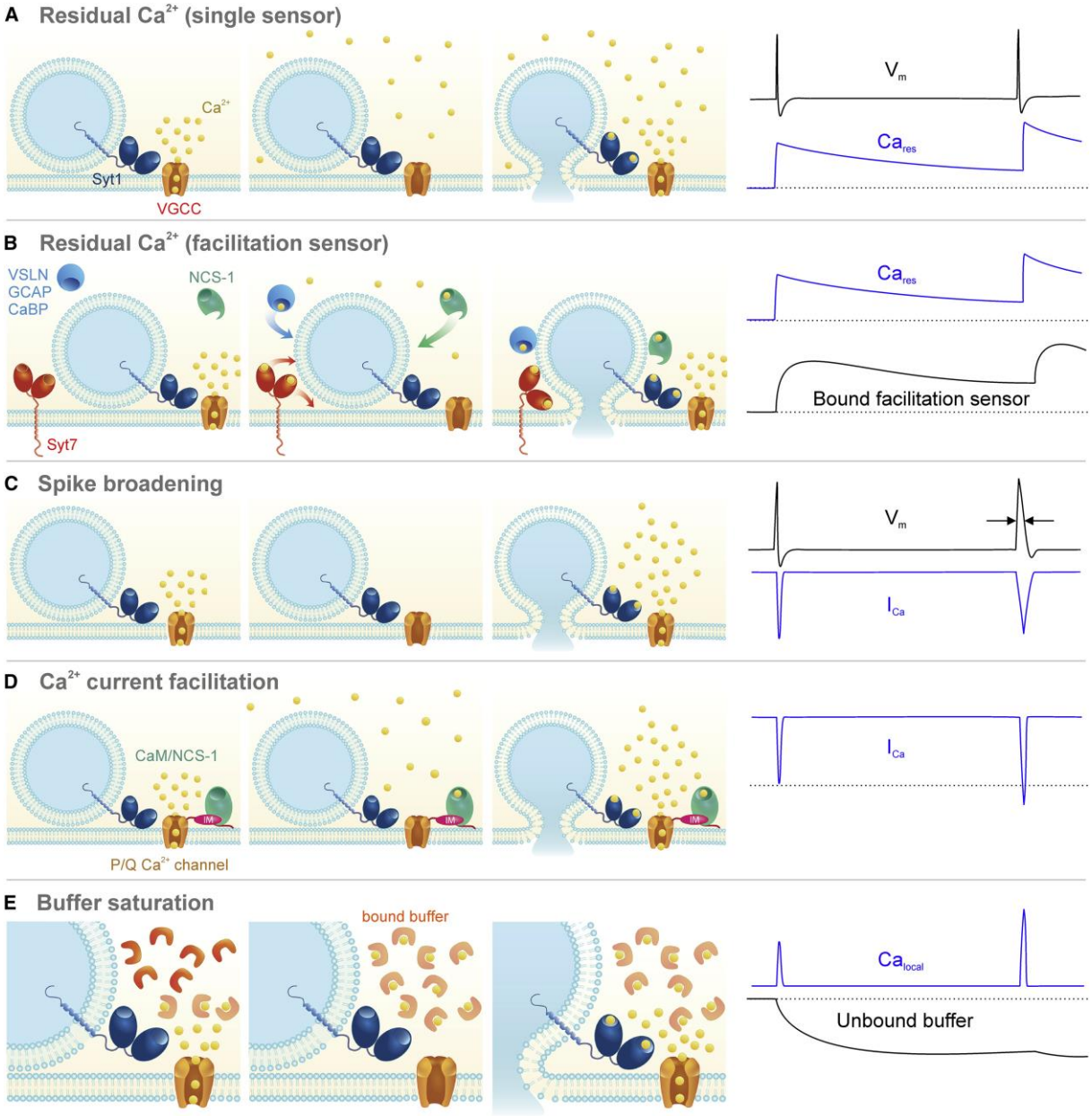

Figure I.3: Mechanisms of synaptic facilitation.
Diagram illustrating proposed mechanisms for facilitation of synaptic vesicle release. The arrival of the action potential (represented as peak and undershoot of the membrane voltage (V_m)) opens voltage-gated calcium channels (VGCCs) and allows for the influx of calcium. In this example, unfacilitated release (left-most panels) fails to activate Synaptotagmin 1 (Syt1) and evoke release.
(*A*) Residual calcium hypothesis proposes that the residual calcium (Ca_{res}) acts in summation with the local calcium (Ca_{local}) to allow for a stronger release in response to the second action potential.
(*B*) Ca_{res} acts on a second sensor (facilitation sensor) to increase release. A proposed facilitation sensor is Synaptotagmin 7 (Syt7, Jackman 2016 Nature).
(*C*) Strong activity can lead to inactivation of K^+ channels, broadening of the action potential waveform and increased calcium entry.
(*D*) Calcium influx from the first action potential can lead to activation of other calcium sensors (like CaM), which modulate calcium channels and increase calcium influx.
(*E*) Ca^{2+} buffers capture some calcium ions from the first action potential, which allows for more free Ca^{2+} during the second action potential.
Reprinted from Neuron, 94, S. Jackman and W. Regehr, 'The Mechanisms and Functions of Synaptic Facilitation', page 452, 2017, with permission from Elsevier.

1.3.1.2 Synaptic Depression

Contrary to facilitation, synaptic depression is the reduction in synaptic strength upon repeated stimulation. Similarly to facilitation, depression is more typically due to presynaptic processes. However, postsynaptic processes can also play a role in short-term depression (Zucker and Regehr, 2002). Desensitization and saturation of postsynaptic receptors have been shown to play a role in reducing transmission (Otis et al., 1996; Neher and Sakaba, 2001; Wadiche et al., 2001) though this effect does not seem to be widespread across the brain in physiological conditions (Zucker and Regehr, 2002).

Several different mechanisms can bring about synaptic depression. One prominent model of depression is that of depletion of the RRP (Fioravante and Regehr, 2011). Upon the arrival of each action potential a fraction of the RRP is released. If a second action potential arrives before new vesicles have replenished the RRP, fewer vesicles will be released. This model explains why depression tends to be stronger in synapses with vesicles with higher P_{vr} and at higher stimulation frequencies.

The equation in page 21 is useful in understanding the depletion model of depression and also its interplay with facilitation. The vesicle consumption during activity leads to a reduction in the release sites occupied by release-ready vesicles. This is translated to a decrease in p_{occ} (Figure I.4). As described in the previous section, activity also leads to an increase in P_{vr} ($=p_{succ}$). The increase in P_{vr}, the decrease in p_{occ}, and the replenishment of vesicles (partially recovering p_{occ}) consist a simple model to explain STP (Figure I.4B, Neher 2017). If P_{vr} is initially high, a great portion of vesicles will be released, leading to a strong reduction in p_{occ}. This will result in depression. On the other hand, if P_{vr} is low, only a small portion of the fusion-competent vesicles will be released. In this case, the increase in P_{vr} (or p_{succ}) can overcome the decrease in p_{occ}, resulting in facilitation. Even though this model does not account for factors such as the heterogeneity of P_{vr} in different vesicles, its simplicity is very useful in understanding the underlying mechanisms of STP.

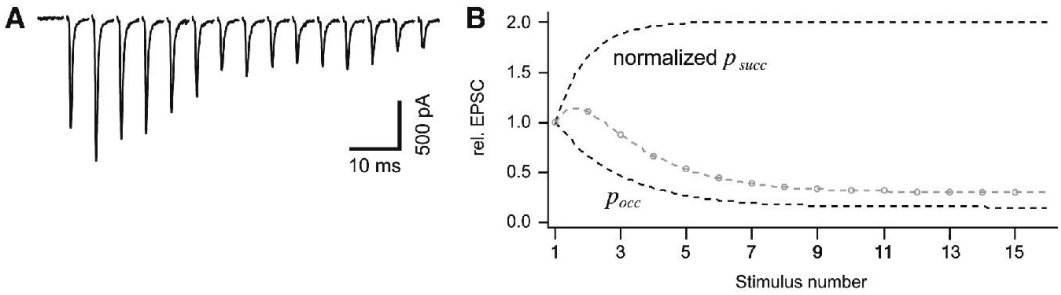

Figure I.4: Dynamic interplay between p_{succ} and p_{occ} can describe short-term plasticity.
(*A*) A sequence of EPSCs recorded in the calyx of Held in response to a train of stimulation at 200 Hz. (*B*) Schematic representation of the estimates of p_{succ} and p_{occ} as well as the normalized EPSC amplitude (*gray circles*) during the train of stimulation. Mechanisms of facilitation lead to an increase in p_{succ} (P_{vr}) during activity, whereas p_{occ} decreases due to vesicle depletion. Reprinted from Biophysical Journal, 112, E. Neher, 'Some Subtle Lessons from the Calyx of Held Synapse', page 216, 2017, open access article under the CC BY-NC-ND license (http:// creativecommons.org/licenses/by-nc-nd/4.0/); DOI: 10.1016/j.bpj.2016.12.017.

Another mechanism that could lead to the depression of a synapse is the inactivation of a release site after a vesicle has just been released. That would mean that the secretion of a vesicle would block subsequent release at that site even if the RRP is not depleted (Neher and Sakaba, 2008). This inactivation could reflect the time necessary to clear the release site of vesicular membrane proteins, present due to vesicle fusion.

Several other proposed mechanisms of depression exist. For example, there can be a dynamic reduction in calcium influx, leading to weaker release. One way how this can be achieved, which is relevant to this work, is by activation of mGluRs. This is the case at the mossy fiber to CA3 pyramidal cell synapse, where activation of mGluRs can abolish release (Kamiya et al., 1996). In this synapse, however, activation of mGluR2 inhibits both calcium influx and the release machinery (Kamiya and Ozawa, 1999).

1.3.1.3 Roles of Short-term Plasticity

Several mechanisms are in place to allow for short-term plasticity. But the question still remains open: what purpose does it serve? There are several proposed roles for this dynamic change in synaptic strength.

One straightforward role of facilitation is to counteract synaptic depression. The increase in P_r generated by facilitation can partially compensate for the depletion of the RRP (Jackman and Regehr, 2017). This is particularly important in some synapses, where

facilitation can increase robustly with firing frequency, offsetting, therefore, the RRP depletion, as it happens in a cerebellar synapse (Turecek et al., 2016).

Another important role, which was already mentioned on page 25, is the temporal filtering. Facilitating synapses often have a low P_r and can be, therefore, unreliable in transmitting single action potentials. However, bursts of activity can greatly increase P_r and, therefore, temporarily convert the synapse into extremely reliable in conveying information to the postsynaptic neuron. In this way, facilitating synapses act as high-pass filters (Lisman et al., 1997). That means that stimuli at low frequencies do not get transmitted forward, while high frequencies strongly influence the post-synaptic terminal. Synapses that undergo depression, conversely, act as low-pass filters (Abbott et al., 1997; Fortune and Rose, 2001). By extension, synapses that can depress or facilitate can act as band-pass filters (Dittman et al., 2000).

These properties, when combined with excitation and inhibition in circuits, can lead to a multitude of ways of how information is transferred and how circuits behave (Jackman and Regehr, 2017). It has also been proposed that facilitation would be sufficient to be the neural correlate of working memory in the prefrontal cortex (Mongillo et al., 2008) or possibly in the CA3 of the hippocampus (Kesner, 2007; Hagena and Manahan-Vaughan, 2010).

1.3.2 Long-Term Plasticity

Functional synaptic plasticity is not confined to short periods of time but can also span many hours. This long-term plasticity can also lead to an enhancement of transmission, i.e. long-term potentiation (LTP), or a reduction in the efficacy of transmission, i.e. long-term depression (LTD). Both of them have been implicated in being of great importance in memory (e.g. Nabavi et al. 2014; Roy et al. 2016; see also Bliss & Collingridge 1993 and Poo et al. 2016).

Typically, LTP or LTD can be evoked by repeated stimulation at specific frequencies or by activating pre- and postsynaptic terminals consecutively within certain time intervals. A common way to induce LTP is with strong stimulation at high frequency. The most studied form of LTP is at the synapses from the Schaffer collaterals onto CA1 pyramidal cells, and its expression is classically described as an increase in AMPA receptor presence in the postsynaptic membrane (Kauer et al., 1988; Muller et al., 1988). This increased receptor

presence leads to the increased synaptic efficacy observed in LTP. This form of LTP is dependent on Ca^{2+} entry through postsynaptic NMDA receptors (Harris et al., 1984; Volianskis et al., 2015).

There are, however, NMDA-independent forms of LTP. The hippocampal mossy fibers display a form of LTP that is mostly expressed presynaptically, through an increase in neurotransmitter release instead of AMPA receptor presence, and is independent of NMDA activation (Harris & Cotman 1986; for a review see Nicoll & Schmitz 2005). A lengthier description about the mossy fibers can be found below (1.4.1 'The Mossy Fibers'), but let's start from the beginning, understanding what is the hippocampus.

1.4 The Hippocampus

The hippocampus, from the Greek word 'ἱππόκαμπος' (seahorse), derives this name because of its anatomical resemblance to such animal. It can be divided in two parts: the hippocampus proper and the dentate gyrus (DG; Figure I.5). The hippocampus proper can also be called Ammon's Horn (in Latin *Cornu Ammonis*), which gives name to its subdivisions: CA1, CA2, CA3 and CA4. CA2 is a narrow region between CA1 and CA3, for which there is limited knowledge, though a growing body of literature seems to have been recently added to findings in this area. CA4 is actually a deep layer of the dentate gyrus. Focus here will then be given to CA1, CA3 and the axonal fibers that connect DG and CA3.

Axonal fibers, known as the perforant path, project from the entorhinal cortex mainly to DG. Some axons also project directly to CA3 and to CA1. The granule cells in DG emit axons called mossy fibers, which synapse onto cells in the CA3. Their main targets are the dendrites of the principal neurons in the CA3, called the pyramidal cells. From there, axons called Schaffer collaterals connect to the dendrites of CA1 pyramidal cells. These cells project axons back to the entorhinal cortex. This is called the trisynaptic circuit because of its three synapses: perforant path to DG, mossy fiber to CA3, Schaffer collaterals to CA1 (Andersen et al., 2007). The last two will be assessed in this thesis.

The hippocampus is a layered structure (Figure I.5C). Stratum oriens can be described as the first layer of the hippocampus proper and it consists of interneurons and, since it contains the basal dendrites of the pyramidal cells, some CA3 to CA3 and CA3 to CA1 connections. The pyramidal cells themselves form the next layer, the stratum pyramidale

(or pyramidal cell layer). Above the pyramidal cell layer is located the stratum lucidum, but exclusively in the CA3. The mossy fibers, originating from DG granule cells, travel through this layer. Because these axons are unmyelinated, they give this layer a clear appearance in fresh tissue, which gives this layer its name (*lucidum*: clear, bright in Latin). The mossy fibers terminate in the border between CA3 and CA2, which explain the exclusivity of the stratum lucidum to CA3. Therefore, in the CA3, the stratum radiatum lies above the stratum lucidum, while in CA2 and CA1 it is located above the stratum pyramidale. In this layer, a variety of interneurons, CA3 to CA1 Schaffer collateral connections and many associational CA3 to CA3 connections are located. It is notable that the CA3 has a large number of recurrent connections. Lastly, superficial to the stratum radiatum is the stratum lacunosum-moleculare. This layer also contains interneurons, as well as terminals from axons originating in the entorhinal cortex as well as some from other brain areas.

The hippocampus has been largely associated with the formation of new memories, mainly episodic memories. These are memories about experienced, or autobiographical, events. The hippocampus is also very important in spatial cognition, having a vital role in spatial coding. It is suggested that the hippocampus harbors the brain's cognitive map (O'Keefe and Nadel, 1978).

Two interesting computational processes, associated with memory and spatial function in the hippocampus, are pattern completion and pattern separation (Rolls and Kesner, 2006). Pattern completion is the process through which one cue, or a small subset of cues, can lead to recall of a whole memory. It is to smell a faint perfume and then remember the day you met your first love. Pattern separation, on the other hand, is the process through which even small differences can lead to different memory representations. It is to be able to find your car every day even though it is parked in a different spot. The CA3 in the hippocampus, in association with DG, has been proposed to be involved in pattern separation and pattern completion (O'Reilly and McClelland, 1994; Guzowski et al., 2004; Gilbert and Kesner, 2006; Rolls, 2013; Guzman et al., 2016; Knierim and Neunuebel, 2016).

There are, therefore, distinctions in function within the different areas in the hippocampus. For example, the CA3 region seems to be indispensable for rapid one-trial contextual learning and pattern-completion memory recall, but not required for incremental spatial learning, for which CA1 is required (Nakashiba et al., 2008).

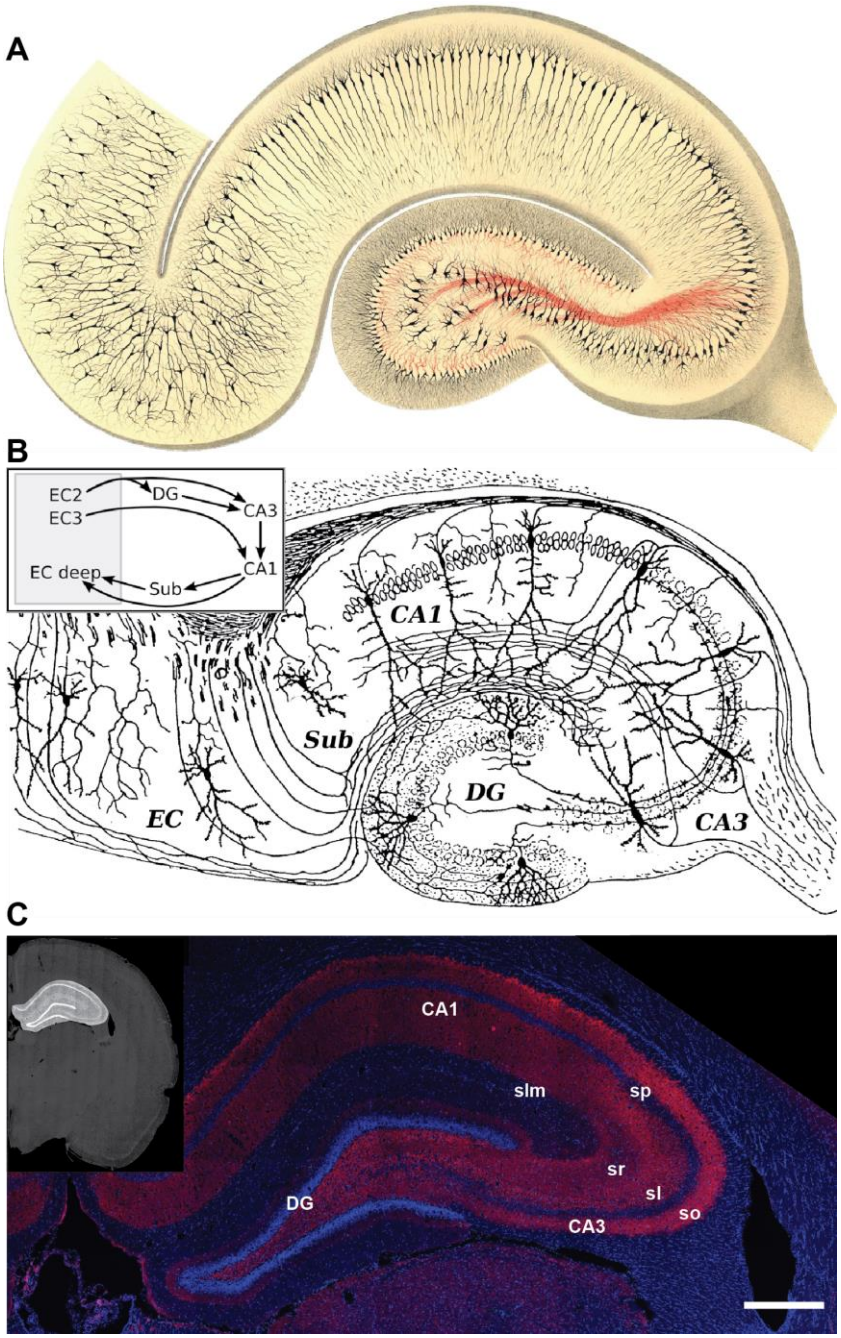

Figure I.5: Representations of hippocampus anatomy, circuitry and layers.
(*A*) Drawing by Camillo Golgi (1903, Public Domain) of a silver nitrate-stained hippocampus. This staining allows for sparse labeling of cells. Pyramidal cells (CA1-3) are easily discernible by their orderly presence on a single layer. Mossy fiber axons are visible in red.
(*B*) Modified drawing by Santiago Ramón y Cajal (1911, Public Domain) representing the basic circuit of the hippocampus.
(*C*) Immunohistochemical staining of a coronal section of rat hippocampus, kindly provided by Rebecca Wallrafen. Section was stained for Mover (red) by antibody binding, and nuclei (blue) by DAPI (4',6-diamidino-2-phenylindole). Inset on top left: coronal section of a rat brain hemisphere, stained by DAPI (4',6-diamidino-2-phenylindole), hippocampus position highlighted. DG: dentate gyrus; EC: entorhinal cortex, EC2: layer II of the entorhinal cortex; EC3: layer III of the entorhinal cortex; EC deep: deep layers of the entorhinal cortex; Sub: subiculum; sl: stratum lucidum; slm: stratum lacunosum-moleculare; so: stratum oriens; sp: stratum pyramidale; sr: stratum radiatum. Scale bar: 500 µm

1.4.1 The Mossy Fibers

The axons of the granule cells in the dentate gyrus form the mossy fibers. They were so named by Santiago Ramón y Cajal because of the presence of varicosities and filopodia along the axons, giving it a "mossy" appearance. The mossy fibers, which are unmyelinated, make excitatory synapses onto hilar mossy cells, interneurons, and pyramidal cells in the CA3 (Andersen et al., 2007). Here, a focus will be given to the synapse formed between the mossy fibers and the dendrites of CA3 pyramidal cells. In this thesis, the mossy fiber to CA3 pyramidal neuron synapse will shortly be referred to as 'mossy fiber synapse'.

The mossy fibers connect to the pyramidal cells via the thorny excrescences, which are intricately branched spines in the proximal dendrites of the pyramidal cells. A granule cell can connect to 11-15 CA3 pyramidal cells, though each mossy fiber terminal only connects to the dendrite of one neuron (Acsády et al., 1998). The mossy fiber terminals form giant (~8µm^3) presynaptic boutons, which, in adult rats, harbor the astonishing amount of 16,000 synaptic vesicles (Figure I.6A; Rollenhagen et al., 2007; Rollenhagen and Lübke, 2010). In young rats, the presence of vesicles is even more overwhelming, with 25,000 vesicles in an even smaller bouton (~7µm^3). A bouton contains in average 30 active zones, each with ~36 vesicles in close association with it (Rollenhagen et al., 2007). Those are supposed to consist the RRP and are, indeed, corroborated by electrophysiological measurements (Hallermann et al., 2003).

This large number of release-ready vesicles (1200-1400 per bouton), coupled with a remarkably low P_{vr}, allows for the strong facilitation observed at this synapse (Hallermann et al., 2003; Lawrence et al., 2004; Rollenhagen et al., 2007). This activity-dependent facilitation allows the mossy fibers to respond dynamically to a wide range of stimuli (Nicoll and Schmitz, 2005). In addition, the mossy fiber terminals are considered "conditional detonators", as they can reliably make the postsynaptic neurons fire, provided a condition is fulfilled: a burst of activity must happen to elicit facilitation, therefore switching the synapse from subdetonators to full detonators (Vyleta et al., 2016).

The STP in mossy fiber synapses is atypical not only because of its strong facilitation, but also because it happens over a wide temporal range. While in many synapses facilitation only lasts hundreds of milliseconds, in the mossy fibers strong facilitation can be observed with stimulation frequencies as low as one stimulus every 10 seconds (Salin et al., 1996; Zucker and Regehr, 2002).

Several factors influence the strong, long-lasting facilitation observed at this synapse. Activity-dependent inactivation of K+ channels in this synapse leads to spike broadening and, consequently, to a stronger rise in Ca^{2+} concentration (Figure I.3C; Geiger & Jonas, 2000). Additionally, saturation of endogenous calcium buffers, in association with a loose coupling between calcium channels and calcium sensors contributes both to keeping a low P_{vr} and to increasing facilitation (Figure I.3E; Blatow et al., 2003; Vyleta and Jonas, 2014). Syt7, acting as a calcium sensor for facilitation, is also required for the observed facilitation at these synapses (Jackman et al., 2016). Activation of presynaptic Kainate receptors by synaptic activity can also lead to stronger facilitation by depolarization of the presynaptic terminal, increase in calcium influx and activation of the cAMP pathway (Schmitz et al., 2000; Kamiya et al., 2002; Scott et al., 2008a; Andrade-Talavera et al., 2012).

Activation of Kainate receptors of mossy fibers is also important for the LTP at this synapse (Lauri et al., 2001a). The mossy fiber synapse expresses a presynaptic form of LTP that is NMDA receptor-independent and requires activation of the cAMP pathway (Figure I.7; Harris and Cotman, 1986; Weisskopf et al., 1994). Nevertheless, this synapse also displays a NMDA receptor-dependent LTP that is expressed postsynaptically (Kwon and Castillo, 2008).

1.4.2 The Schaffer Collaterals

The pyramidal cells in the CA3 give off highly collateralized axons. These connect to the ipsilateral and contralateral hippocampi as well as to the lateral septal nucleus (Andersen et al., 2007). The collaterals that connect to the pyramidal cells in the CA1 are called the Schaffer collaterals. The Schaffer collaterals are one of the most studied pathways in the brain, in part due to its layered organization and rather simple circuitry, and because of the extensive study of LTP in their synapses (Nicoll, 2017).

STP, on the other hand, is not very prominent on the Schaffer collaterals, especially when compared to the mossy fibers. While a paired-pulse ratio produces a somewhat similar degree of facilitation in both synapses, a short train of stimulation leads to only a modest increase in the Schaffer collateral responses, facilitation in the mossy fiber to CA3 pyramidal cell synapses can be 10-fold stronger (a direct comparison can be seen in Jackman et al., 2016).

This difference in STP is also reflected in the different composition of this synapse. The synapse between Schaffer collateral and CA1 pyramidal cell is, in many ways, considered to be a rather "typical" synapse, with one active zone per synapse, 10 docked vesicles per active zone and a total pool of 200 vesicles per synapse (Figure I.6B; Schikorski and Stevens, 1997). Furthermore, the ~10 docked vesicles are believed to represent the synapse's RRP (Schikorski and Stevens, 2001).

Axonal collaterals from a single CA3 pyramidal cell can connect to 30,000-60,000 neurons (Li et al., 1994). Besides the Schaffer collaterals, which project to CA1, other collaterals enervate the CA3 of the ipsilateral or the contralateral hippocampi. These are called associational or commissural fibers, respectively.

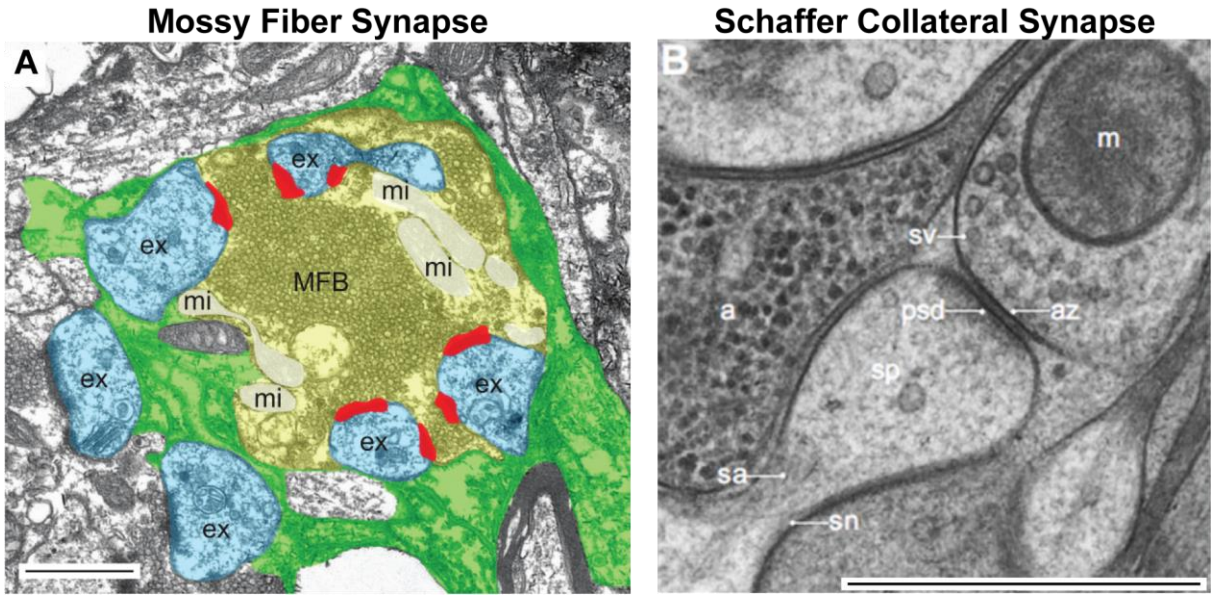

Figure I.6: Electron micrographs illustrating the mossy fiber and Schaffer collateral synapses.
(*A*) Mossy fiber bouton (*MFB*, *yellow*) in the CA3, with several active zones (*red*) connecting it to the postsynaptic thorny excrescences (*ex*, *blue*) of a pyramidal cell. The surrounding glia is marked in green and the mitochondria (*mi*) within the MFB in white. Note that every small dot inside the MFB represents one vesicle. Reprinted from Frontiers in Synaptic Neuroscience, 2, A. Rollenhagen and J. Lübke, 'The mossy fiber bouton: The "common" or the "unique" synapse?', page 2, 2010 open access.
(*B*) CA1 stratum radiatum glutamatergic synapse onto a dendritic spine (sp), from an organotypic hippocampal slice culture. Synaptic vesicles (*sv*) are visible between the mitochondrion (*m*) and the active zone (*az*). Opposite to the active zone is the post-synaptic density (*psd*). The spine neck (*sn*) and apparatus (*sa*) are also visible in the dendrite. Next to the synapse an astrocyte (*a*) is present. Note the considerably smaller and simpler bouton and fewer vesicles when compared to the mossy fiber bouton. Reprinted with permission of Springer from 'Synapse Development: Methods in Molecular Biology', 'Chapter 15: 3D Analysis of Synaptic Ultrastructure in Organotypic Hippocampal Slice Culture by High-Pressure Freezing and Electron Tomography' by C. Imig and B.H. Cooper, page 225, © Springer Science+Business Media LLC 2017.
Scale bars: 1 µm.

1.5 Mover

Mover is a vesicle-attached, vertebrate-specific protein that is present in neurons (Ahmed et al., 2013). It consists of 266 amino acids, weights approximately 30 kDa, and it was found in a yeast-2-hybrid assay using Bassoon as bait (Kremer et al., 2007). The same protein was found in a proteomic analysis of synaptic vesicles and was named Synaptic vesicle-associated protein of 30 kDa (SVAP30; Burré et al., 2006). A third study found the same very protein as a homolog of a target of the transcription factor p63 and named it Transformation related protein 63 regulated like (Tprgl; Antonini et al., 2008).

Mover has a high expression in the brain, but also in heart, liver, kidney and testis (Kremer et al., 2007; Antonini et al., 2008). In the brain, it localizes to presynaptic terminals, which fits its association with synaptic vesicles (Burré et al., 2006; Kremer et al., 2007; Ahmed et al., 2013). Its distribution is not homogeneous to all synapses in the brain, but is enriched in some areas such as the stratum lucidum of the hippocampus, the brainstem synapses called calyx of Held and the inhibitory synapses in the cerebellum. On the other hand, it seems mostly absent from inhibitory synapses in the hippocampal CA3, and from excitatory synapses in the cerebellar cortex (Kremer et al., 2007).

An attribute of Mover that is required to target it to presynaptic terminals is that it self-interacts (Ahmed et al., 2013). It is likely that the hSac2 domain in Mover is important for its dimerization (Hsu et al., 2015). This domain is, however, not sufficient for Mover self-interaction and targeting to synapse, as many other parts of the protein are also required (Akula, 2015).

Furthermore, the phosphorylation of Mover is required for its association to synaptic vesicles (Ahmed et al., 2013). Additionally, the phosphorylation of Mover is not regulated by synaptic activity in synaptosomes (Kohansal-Nodehi et al., 2016), but its site S14 can be dephosphorylated by Calcineurin (Protein phosphatase 2B; Mahdokht Kohansal-Nodehi personal communication). Since the activity of Calcineurin is Ca^{2+}/CaM dependent (Rusnak and Mertz, 2000), this can confer a possible activity-regulated de/phosphorylation of Mover.

Notably, neuronal activity can also regulate the expression of Mover. Mover levels were decreased in neuronal cultures upon blockade of activity by tetrodotoxin (TTX; Kremer, 2008) and in an auditory synapse upon disruption of the upstream synapse (Wetzel, 2015).

Consistently, Mover expression was increased by the Adenylyl Cyclase activator forskolin (Moritz Arndt, personal communication). Furthermore, Mover presence in vesicles was increased in brains of rats perfused with a solution with high K^+ concentration (Burré et al., 2006). Curiously, increased Mover levels have also been found in the Anterior Cingulate Cortex of schizophrenic patients (Clark et al., 2006).

Knockdown of Mover in the calyx of Held revealed an acceleration in vesicle reloading after synaptic depression and an increase in P_{vr}, with no effect on the RRP (Körber et al., 2015). This increase in P_{vr} was not caused by a stronger calcium influx but by a boost in the calcium sensitivity of release. Since the observed change is best explained by an increase in the intrinsic Ca^{2+} sensitivity of the release sensor, it was linked to superpriming (Körber and Kuner, 2016). Additionally, in a knockout of Bassoon, P_{vr} was increased in association with a reduction in synaptic Mover (Mendoza Schulz et al., 2014).

Besides binding to Bassoon, Mover has also been shown to bind to CaM in a Ca^{2+}-dependent manner (Körber et al., 2015). I will now give a brief introduction on these two binding partners of Mover.

1.5.1 Bassoon

Bassoon is a vertebrate-specific, 420 kDa protein, and a component of the cytomatrix of the active zone (tom Dieck et al., 1998; Gundelfinger and Fejtova, 2012). Besides interacting with Mover (Kremer et al., 2007), Bassoon also binds to other proteins that influence presynaptic activity, such as Munc13-1 (Wang et al., 2009), RIM (Wang et al., 2009), CAST/ELKS (Takao-Rikitsu et al., 2004), CtBP1 and CtBP2/RIBEYE (tom Dieck et al., 2005). Bassoon also binds Piccolo and is trafficked together with it to active zones via Golgi-derived vesicles (Dresbach et al., 2006; Maas et al., 2012).

Importantly, Bassoon, as well as RIM, indirectly binds to VGCCs through RIM-BP (Hibino et al., 2002; Han et al., 2011; Kaeser et al., 2011; Davydova et al., 2014). Accordingly, Bassoon has a role in Ca^{2+} channel clustering in ribbon synapses (tom Dieck et al., 2005; Frank et al., 2010). Loss of Bassoon in ribbon-type synapses can lead to a reduction in the RRP and impairment of vesicle replenishment (Frank et al., 2010). In these synapses, Bassoon is necessary for the proper positioning of the ribbon and is, therefore, proposed to act as a tether between the ribbon and the active zone (Khimich et al., 2005; Gundelfinger et al., 2016).

In non-ribbon synapses, Bassoon has also been proposed to act as a vesicle tether (Hallermann et al., 2010; Hallermann and Silver, 2013). Accordingly, disruption of Bassoon in the cerebellar mossy fiber to granule cell synapse and in the endbulb of Held also led to a slowdown in vesicle replenishment (Hallermann et al., 2010; Mendoza Schulz et al., 2014). In the endbulb of Held, the slower replenishment was also accompanied by a reduction in the RRP. Additionally, increases in the postsynaptic densities, in the quantal size and in P_{vr} were also observed, but are possibly due to homeostatic adaptation. It is also possible that the increase in P_{vr} was brought about by a reduction in Mover levels (Mendoza Schulz et al., 2014).

Bassoon, together with Piccolo, is also involved in synapse maintenance and integrity by maintaining synaptic vesicle clustering (Mukherjee et al., 2010), and regulating the ubiquitin-proteasome system (Waites et al., 2013). Moreover, Bassoon can also control presynaptic autophagy (Okerlund et al., 2017).

1.5.2 Calmodulin

In contrast to the vertebrate-specificity of Mover, Calmodulin (CaM) can be found across the eukaryote domain and its amino acid sequence is extraordinarily conserved from yeast to humans (Ikura and Ames, 2006). CaM has a central role in sensing calcium, as it binds more than 350 presumed targets, though it can also bind to proteins in a Ca^{2+}-independent way (Calmodulin Target Database: Yap et al., 2000).

CaM has several functions in the synapse, both in the postsynaptic terminal as well as in the presynaptic. In the presynapse, it can drastically affect STP due to its binding to Munc13 (Junge et al., 2004). When the binding between CaM and Munc13-2 is prevented, stimulation that would usually lead to facilitation leads to depression instead; and in the isoform bMunc13-2, this effect is accompanied by a strong increase in P_{vr} and RRP (Junge et al., 2004; Lipstein et al., 2012). Furthermore, CaM also regulates the refilling of the RRP (Sakaba and Neher, 2001a). It does so by a Ca^{2+}-driven acceleration of vesicle priming through its binding to Munc13-1, -2 or -3 (Junge et al., 2004; Lipstein et al., 2012, 2013). Curiously, this effect can be dependent on cyclic adenosine monophosphate (cAMP) levels, as activation of $GABA_B$ receptors slows down the recruitment of synaptic vesicles to the RRP (Sakaba and Neher, 2003).

In cultured neurons, knockdown of Calmodulin leads to a strong decrease in P_{vr} without altering the RRP size (Pang et al., 2010). This effect is dependent on Calmodulin-dependent Kinase IIα and is independent of activity or residual calcium levels (i.e. initial response after a period of quietude was already affected). Additionally, CaM is also proposed to be involved in asynchronous release as an auxiliary Ca^{2+} sensor (Sun et al., 2007).

Neuromodulin is proposed to regulate levels of free CaM in conjunction with Protein Kinase C (Figure I.7; Xia and Storm, 2005). When free, CaM, together with Ca^{2+}, can bind and stimulate some isoforms of Adenylyl Cylase (AC): AC1, AC3 and AC8 (Halls and Cooper, 2011). Interestingly, deletion of AC1 or AC8 impairs LTP in the mossy fibers (Villacres et al., 1998; Wang et al., 2003). The synthesis of cAMP by AC influences LTP by interacting with Rab3A, through PKA and RIM1α (Figure I.7; Castillo et al., 1997, 2002; Lonart et al., 1998).

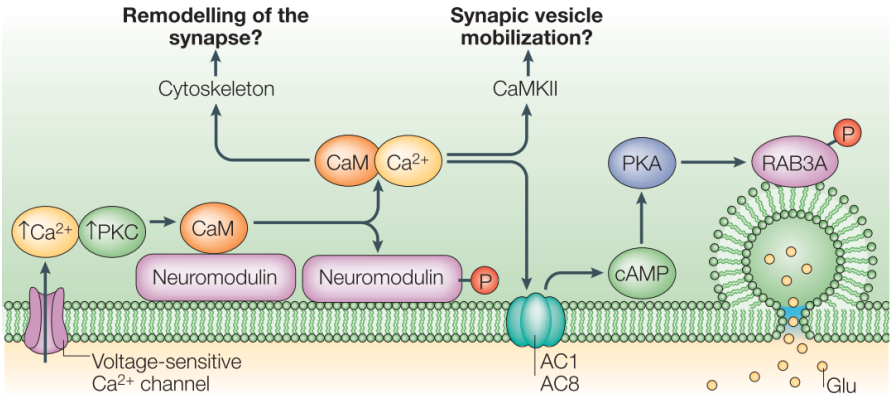

Figure I.7: Influence of Calmodulin in presynaptic long-term potentiation.
Increase in Ca^{2+} levels and activation of PKC (Protein Kinase C) allow Neuromodulin to release bound CaM (Calmodulin). Ca^{2+}/CaM can stimulate certain forms of AC (Adenylyl Cyclase), such as AC1 and AC8. AC synthesizes cAMP (cyclic adenosine monophosphate), which activates PKA (Protein Kinase A). PKA stimulates Rab3A, possibly through RIM1α (not shown). This mechanism leads to an increase in neurotransmitter release and is important for presynaptic forms of LTP such as in the hippocampal mossy fibers. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, 6, Z. Xia and D. R. Storm 'The Role of Calmodulin as a Signal Integrator for Synaptic Plasticity', page 270, © 2005.

1.6 Aim of this study

It was shown that the knockdown of Mover in the calyx of Held leads to an increase in calcium-sensitivity of release and short-term depression (Körber et al., 2015). This knockdown study is so far the only study testing the function of Mover at synapses. My PhD project is the first to analyze in detail the effect of Mover knockout. It aims to understand Mover's role in neurotransmission in synapses in the hippocampus, which have a strong expression of this protein and distinct types of plasticity. To perform this study, I established extracellular and intracellular electrophysiological recordings in acute hippocampal slices, as well as dissociated hippocampal neuronal cultures, of wildtype and Mover knockout mice in the lab. In particular, my study seeks to clarify the mechanisms through which Mover affects presynaptic function and short-term plasticity at hippocampal synapses. In addition, these insights should also shed light onto the general role of this vertebrate-specific protein in the evolutionarily conserved presynaptic machinery.

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2 MATERIAL AND METHODS

2.1 Animals

For the purpose of this study wildtype (WT) and Mover knockout (KO) littermate mice (*Mus musculus*) were bred and maintained at the central animal facility of the University Medical Center, Göttingen. Generation of knockout mice has been described in the theses of Dr. Asha K. Akula (Akula, 2015) and Dr. Friederike Wetzel (Wetzel, 2015). Mice were maintained through heterozygous breeding and routinely genotyped, and regenotyped post experiments. Mice were treated according to the specifications of the University of Göttingen and the state of Lower Saxony (Landesamt für Verbraucherschutz, Braunschweig, Germany).

For genotyping DNA was extracted from tail-cuts, using Nexttec DNA extraction kits (Nexttec Biotechnologie, Germany). Extracted DNA was then amplified by polymerase chain reaction (PCR) using the following reagents: 1µL dimethyl sulfoxide (DMSO), 1µL DNA, 7.5µL H_2O, 12.5 µL Hot Start Green PCR Master Mix (2X; Thermo Scientific) and 1µL of each primer, here named B008-P4, E-3001 and E-4001 and described below (Table 1). PCR was run in a T3000 thermocycler (Biometra, Germany) following the conditions described in Table 1. Genotyping was performed by Irmgard Weiß and by me.

Table 1: Primers used and PCR strategy

Primers used:		
B008-P4**: CCAATCACAAGGCGAACGAG**		
E-3001**: CATTCAGTGGGACAAGCAGA**		
E-4001**: CAAGGCTCTCCTGATCCAAG**		
PCR conditions:		
Temperature:	Time (min):	Repetitions:
95°C	03:00	1x
95°C	00:30	35x
56°C	01:00	
72°C	01:00	
72°C	07:00	1x

The PCR products were then transferred to a 1% agarose gel made visible through fluorescence with the use of nucleic acid staining solution Midori Green (Biozym, Germany). The gel was run through electrophoresis for 60-90 min at 90V. The expected resulting bands in the gel were as follows: WT 867 bp, KO 697 bp, floxed allele 1106 bp, heterozygous 697 bp and 867bp.

2.2 Primary Neuronal Culture

For work in dissociated cultures I prepared hippocampal neuronal cultures from WT and KO mice of postnatal day 0 (P0). The cultures were prepared in a similar fashion to the method described by Burgalossi et al. (2012), but with several modifications for dissociated instead of autaptic cultures. Namely, hippocampi were dissected from newborn mice in cold Hank's Balanced Salt Solution (Gibco, Thermo Fischer Scientific) and digested by papain (25 U/ml) for 60 minutes at 37°C under gentle agitation (450 rpm). Digestion was then stopped by substitution of the papain solution by a trypsin inhibitor-based stop solution and hippocampi were incubated in this solution for 15 minutes at 37°C with gentle agitation (450 rpm). Washing out of stop solution was followed by trituration of the tissue using a 200 µL pipette tip. Neurons were then counted and plated at a density of 25,000 cells per well in a 24 well-dish filled with Neurobasal A medium supplemented

with B27, GlutaMax and penicillin/streptomycin (0.5ml/L) (all from Gibco, Thermo Fischer Scientific).

2.3 Immunocytochemistry

Hippocampal neuronal cultures from WT and KO mice were fixed after 17 days in vitro with 4% paraformaldehyde. Fixed cells were permeabilized by a phosphate-buffered saline containing 0.3% Triton X-100 and had unspecific antibody binding blocked by the presence of 10% fetal calf serum and 2% bovine serum albumin. Primary antibodies were incubated overnight at 4^{o}C. Secondary antibodies were incubated for 1 hour at room temperature in a similar solution but without fetal calf serum. The antibodies used in this thesis are described below (Table 2). Stainings were visualized by epifluorescence through a Zeiss Observer Z1 inverted microscope (Zeiss, Germany).

Table 2: Antibodies for Immunocytochemistry

	Antibody	Host	Dilution	Company
Primary	Mover	rabbit	1:1000	Synaptic Systems, Germany
	Bassoon	mouse	1:1000	Enzo Life Sciences, USA
Secondary	Cy3	donkey anti-mouse	1:1000	Dianova, Germany
	Cy5	donkey anti-rabbit	1:1000	Dianova, Germany

2.4 Slice Preparation

For slice electrophysiology 400 mm thick acute transverse hippocampal slices were prepared from male and female mice 18-22 days old unless otherwise noted. Mice were anaesthetized using isofluorane and killed by decapitation. The isolation of the hippocampi was followed by their slicing in a HM650V vibrotome (Thermo Scientific) in a cutting solution containing (in mM): 215 sucrose, 2.5 KCl, 20 glucose, 26 $NaHCO_3$, 1.6 NaH_2PO_4, 1 $CaCl_2$, 4 $MgCl_2$, and 4 $MgSO_4$. A list of all chemicals and where they were purchased from can be found in Table 3. After sectioning, the slices were incubated for thirty minutes in a solution comprised of 50% cutting solution and 50% recording solution. Recording solution for extracellular recordings, henceforth referred to as artificial cerebrospinal fluid (ACSF) contained (in mM): 124 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 2.5 $CaCl_2$, 1.3

$MgSO_4$, and 10 glucose; 300 mOsm/kg. Recording solution for whole-cell patch clamp recordings, henceforth referred to as 4/4 Ringer, was comprised of (in mM): 119 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 4 $CaCl_2$, 4 $MgSO_4$, and 10 glucose; 300 mOsm/kg. The use of 4/4 Ringer improves the recordings from CA3 pyramidal neurons because of three effects: reduction in vesicle release probability, hyperpolarization of neurons due to surface charge screening and increased block of NMDA receptors. The consequence of these effects is a reduction in excitotoxicity and in recurrent CA3 activation. After the aforementioned 30 minutes incubation, the mixed solution was changed to 100% recording solution. After changing of solution, slices were incubated for at least 60 min at room temperature. All solutions were continuously gassed with carbogen (95% O_2, 5% CO_2).

2.5 Electrophysiology

Postsynaptic potentials and currents were recorded using an EPC-10 USB amplifier (HEKA, Germany) connected to a chlorided silver wire in a patch-type pipette, made from borosilicate glass filaments (GB150F-8P, Science Products, Germany). Pipettes were freshly pulled on the day of recording using a P-1000 Pipette Puller (Sutter Instruments, USA). The slices were visualized during the experiments using an Olympus BX51WI upright microscope with either a 5x dry or a 40x water immersion objective. The use of the 40x objective was coupled with differential interference contrast microscopy to allow for better visualization of cells. During experiments, samples were kept at 27.0°C ± 0.5°C by use of an inline solution heater and a chamber heater in a submersion-type recording chamber, and were continuously perfused at 1.0-1.5 ml/min with ASCF.

For recording of Schaffer collateral responses both recording and stimulation electrodes were placed in the stratum radiatum of the CA1, with stimulation electrode being rostral to the recording electrode. For recording of mossy fiber responses the recording electrode was placed at the stratum lucidum of the hippocampus and the stimulation electrode was placed at the border between the dentate gyrus and the hilus (see Figure I.5 for a description on the anatomy of the hippocampus).

At the end of each mossy fiber experiment, to ensure that the responses were not contaminated by associational/commissural inputs, 1 µM of the group II metabotropic glutamate receptor agonist DCG-IV (2S,20R,30R-2-[20,30-dicarboxycyclo-propyl]glycine) was applied to the bath to block mossy fiber responses selectively (Kamiya

et al., 1996). Such procedure was used for both extracellular and intracellular recordings in which there was stimulation of the mossy fibers. Only synaptic responses that were reduced by more than 80% were included in the analysis.

For each sample, every recording was repeated at least 3 times and traces were averaged. Data was sampled at 20 kHz or 50 kHz and low-pass filtered at 2.9 kHz.

2.5.1 Extracellular Electrophysiology Recordings

For recording of field excitatory postsynaptic potentials (fEPSP) the recording pipette (0.5-1.5 MOhm pipette resistance) was filled with 1 M NaCl. Synaptic activity was evoked by electrical stimulation delivered by a linear stimulus isolator (Model A395, World Precision Instruments, USA) connected to a chlorided silver wire in a patch-type pipette with a broken tip (0.2-0.5 MOhm pipette resistance). In most recordings stimulation strength was kept to a minimum (less than 100 μA) to avoid eliciting population spikes that would contaminate the recording of fEPSPs. This way it was possible to reliably measure fEPSP amplitudes that were only minimally influenced by population spikes. For recordings of input-output curves, where higher stimulation strength was required, the parameter tested was the slope of the fEPSP instead of amplitude.

2.5.2 Intracellular Electrophysiology Recordings

For whole-cell recordings the recording pipette (2.0-4.0 MOhm pipette resistance) was filled with a solution containing (in mM): 123 Cs-gluconate, 8 NaCl, 10 HEPES, 10 Glucose, 10 BAPTA, 5 ATP-Mg, 0.4 GTP-Na; 300 mOsm/kg, pH 7.2. It is noteworthy to point out that 10mM BAPTA was included to prevent desensitization of NMDA receptors. This allows for a more precise measurement of changes in short-term plasticity (Tong et al., 1995).

Stimulation of axons was delivered through the use of an isolated current stimulator (Model DS3, Digitimer, UK) connected to a chlorided silver wire in a patch-type pipette (0.2-0.5 MOhm pipette resistance). Cells were clamped at -70 mV for recordings of spontaneous activity and at 30mV for recordings of NMDA postsynaptic currents. Series and input resistances were monitored during recordings with a -5 mV hyperpolarizing pulse 80 ms long. In recordings from cell cultures, neurons were recorded after 14-16 days in vitro and recordings with more than 15 MOhm were discarded from the analysis. In

recordings in CA3 pyramidal cells from acute slices, cells with a series resistance higher than 25 MOhm were not used. Stimulations that resulted in failures were also excluded from the analysis.

Table 3 - Reagents and drugs

Chemical	Company
ATP-Mg	Sigma-Aldrich
BAPTA	Sigma-Aldrich
$CaCl_2$	Sigma-Aldrich
Cesium-gluconate	Kindly provided by Prof. Tobias Moser
DCG-IV	Tocris Bioscience
Forskolin	Sigma-Aldrich
Glucose	BioFroxx, Applichem
GTP-Na	Sigma-Aldrich
HEPES	Sigma-Aldrich
KCl	Sigma-Aldrich
$MgCl_2$	Applichem
$MgSO_4$	Roth
NaCl	BioFroxx, AppliChem
NaH_2PO_4	AppliChem
$NaHCO_3$	AppliChem
NBQX	Abcam
Picrotoxin	Sigma-Aldrich
Sucrose	BioFroxx
TTX	Tocris Bioscience

2.6 Data analysis and statistics

For analysis of evoked electrophysiological data I wrote custom procedures in IgorPro (Wavemetrics, USA) for peak detection of excitatory postsynaptic currents (EPSCs), fEPSPs and fiber volley; calculation of fEPSP and fiber volley slope; and statistical analysis. Peak amplitude was calculated as the average of the five sampled points

surrounding the minimum (or maximum, in case of NMDA EPSCs) in order to minimize variability due to noise. Slope was measured for 0.5 ms of linear part of fEPSP. For extracellular recordings the traces underwent Gaussian smoothing with 10 iterations before analysis. For recordings of low-frequency facilitation the first 7 responses after the change of frequency were discarded so that only the plateau phase was taken into account for the extent of facilitation. Spontaneous electrophysiological data was analyzed using the software MiniAnalysis (Synaptosoft, USA). The threshold for detection of miniature postsynaptic currents was set to 9 pA.

Imaging data was analyzed using the Fiji image processing package of ImageJ (Schindelin et al., 2012). Processing of images was done in the following manner: three regions of interest were selected in regions without any fluorescence signal to measure pixel intensities at background levels. The mean pixel intensity and standard deviation was calculated from the summation of the three regions. The background was considered as the mean plus one standard deviation and was subtracted from the image. Intensity values were normalized, in terms of percentage, to mean Mover intensities of WT animals.

Further statistical analyses were performed with the software GraphPad Prism (GraphPad Software, USA). Statistical significance was verified using unpaired two-tailed Student's t-tests except where otherwise noted. For experiments in neuronal cultures "N" refers to number of cultures in which the experiments were independently repeated and "n" refers to the total number of cells or images. For experiments in acute slices "N" refers to the number of animals and "n" refers to the number of slices (in extracellular recordings) or cells (in whole-cell recordings). Stimulation artifacts were digitally removed in traces representing evoked electrophysiological data. Data is expressed in text and in graphs in terms of mean ± standard error of the mean (SEM).

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3 RESULTS

3.1 Mover knockout does not change spontaneous transmission in hippocampal neuronal culture

An initial electrophysiological characterization of Mover disruption has been assessed in a knockdown scenario (Körber et al., 2015) but never in a knockout. I initiated the investigation with a simple model to estimate if there were changes in spontaneous vesicular release of neurotransmitters. The chosen model was dissociated neuronal hippocampal cultures. It was a good starting point to investigate possible changes in, for example, synapse number, postsynaptic receptor density, and the kinetics of resulting currents after neurotransmitter binding to postsynaptic receptors. Establishing Mover KO neuronal cultures also allowed for other parallel investigations using these cultures for immunocytochemistry and biochemical experiments.

I used these neurons to record miniature excitatory postsynaptic currents (mEPSCs) in dissociated cultures in the presence of 1 µM tetrodotoxin (TTX). The presence of 1 µM TTX blocks voltage-gated sodium channels and therefore prevents the propagation of action potentials. All the postsynaptic currents that can be detected in this situation come from the spontaneous release of neurotransmitter-laden vesicles. Stochastically, only one vesicle would be released at a certain moment. This allowed me to record postsynaptic responses to the release of single vesicles and evaluate whether the knockout of Mover had an effect on that. There were no significant differences between WT and KO neurons in any of the parameters tested (Figure 1). The absence of change in the amplitude of

mEPSCs (Figure 1C; WT: 27.1 ± 1.1nA *n*=31 *N*=4; KO: 28.8 ± 1.2nA *n*=28; *N*=4; *P*=0.29) means that the quantal size is unchanged. This indicates no difference in the presence of postsynaptic receptors or in the amount of neurotransmitter loaded into the vesicles. Lack of change in the frequency of events (Figure 1D; WT: 1.8 ± 0.2 Hz; KO: 1.7 ± 0.3 Hz; *P*=0.67) indicates no difference in the number of synapses or drastic changes in release probability. There was also no difference in the kinetics of mEPSCs, with both rising phase (Figure 1E; 10-90 % rise time; WT 1.3 ± 0.08 ms; KO 1.4 ± 0.09 ms; *P*= 0.34) and decay (Figure 1F; time constant of exponential decay; WT 6.8 ± 0.35 ms; KO 7.6 ± 0.33 ms; *P*=0.07) having comparable values between WT and KO. These data suggest that the knockout did not affect spontaneous release and it also did not promote any major changes in the postsynaptic terminal.

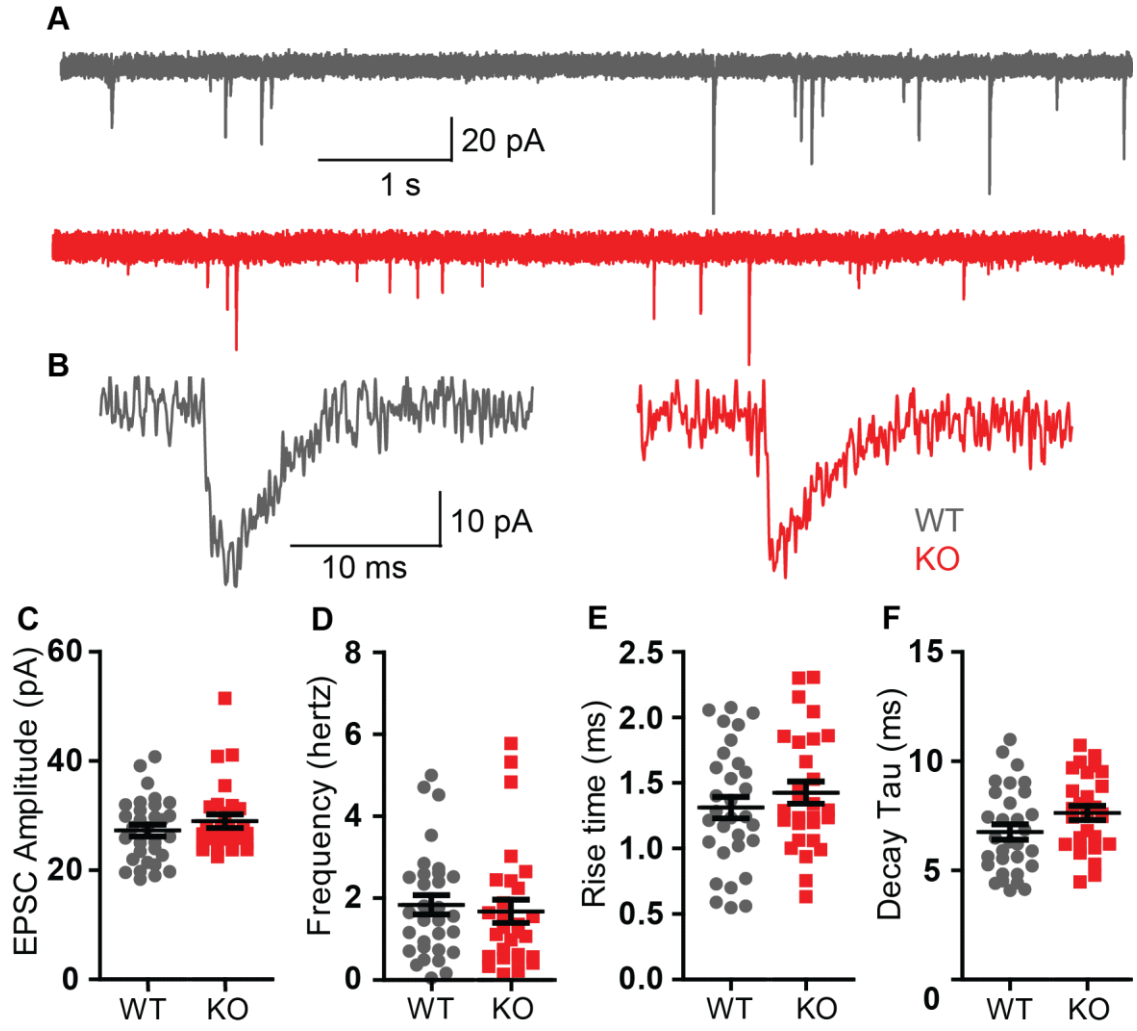

Figure 1: Spontaneous transmission in cultured neurons is unchanged in the absence of Mover.

Whole cell recordings of miniature EPSCs in hippocampal dissociated cultures. (*A-B*) Representative current traces of mEPSC recordings in WT and Mover KO cultured neurons. (*C*) mEPSC amplitude, (*D*) frequency, (*E*) 10-90 rise time, and (*F*) time constant of decay are unchanged. Error bars represent SEM.

After using them for electrophysiology, I fixed and stained the WT and KO cultures. There were two important reasons for these immunocytochemistry experiments. Firstly, I wanted to ensure the specificity of the anti-Mover antibody for future experiments. Secondly, I wanted to verify, using a different method, that Mover was indeed knocked out, absent in the cultures believed to be KO and present only in the WT. Mover was indeed absent in the KO and the cultures had been correctly labeled. As for the specificity of the antibody, fluorescence intensities in Mover KO were reduced by more than 80 % (Figure 2; 83.4 ± 1.8 %; *n*=12-13; *N*=3). The specificity of the antibody seems satisfactory since the remaining 17 % fluorescence can be explained by autofluorescence of the organic matter and minimal unspecific binding. Autofluorescence should be taken into consideration due to the fact that background subtraction was based on intensity levels where there was no presence of neuronal soma or neurites.

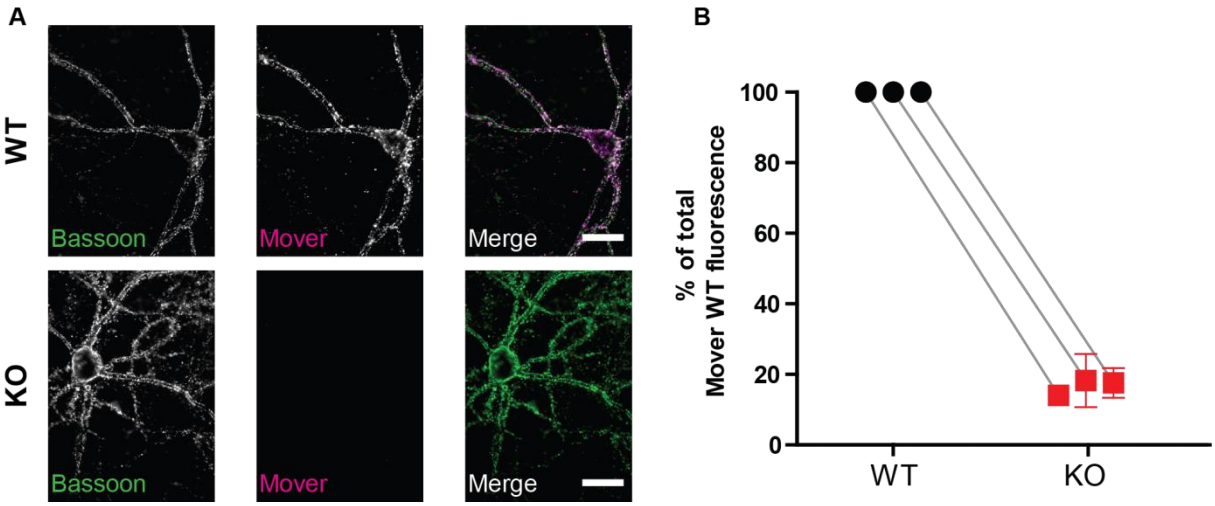

Figure 2: Knockout of Mover confirmed by immunofluorescence.
(*A*) Epifluorescence example images from WT (*top*) and Mover KO (*bottom*) cultures. Cultures were stained for the active zone marker Bassoon as well as for Mover. (*B*) Mover fluorescence intensities in KO are less than 20 % of those in WT. Each data point represents one culture, i.e. one independent experiment comprised of several images taken. Connecting lines pair WT and KO values of the same experiment. Fluorescence intensities levels were normalized to WT intensities for each experiment. Stainings and image capture were performed by Sebastian Molina Obando and supervised by me. Error bars represent SEM. Scale bars=30 µm.

3.2 Mover knockout does not affect synaptic transmission at Schaffer collateral synapses

Due to the observation of high levels of Mover in certain areas of the Hippocampus, I decided to verify the effect of the knockout using field recordings in acute hippocampal slices. I started by analyzing the synaptic transmission at one of the most well studied pathways in the brain: the Schaffer collaterals. To this end, I stimulated the Schaffer collaterals and recorded responses in the stratum radiatum of the CA1 (Figure 3A). By using increasing stimulation currents I was able to analyze the input-output behavior in the absence of Mover. The slope of the fEPSPs was measured at different stimulation strengths. The value of the slopes was then plotted and underwent a linear regression in the linear part of the curve for each sample. Linear regressions are depicted by the following line function:

$$Y = aX + b$$

where 'a' is the slope of the linear fit and 'b' is the Y-intercept. The fits did not vary significantly between WT and KO (Figure 3B). The slope of the fit had values of 11.4 ± 1.9 V/mC (n=13, N=5) for WT and 9.8 ± 1.0 V/mC (n=13, N=4) for KO and were, therefore, not significantly different (P=0.48). Likewise, the Y-intercept was similar for WT (-0.004 ± 0.035 mV/ms) and KO (-0.031 ± 0.029 mV/s; P=0.56). This means that for any given stimulation intensity a similar postsynaptic response was achieved in WT and KO. An extrapolation of this statement is that there were no significant changes in the excitability or P_r of the Schaffer collaterals, or in the distribution of postsynaptic receptors.

The lack of change observed in the input-output curves was a surprise since data obtained from Mover knockdown in the calyx of Held had shown an increase in vesicular release probability (Körber et al., 2015). It is possible, however, that the release was changed but homeostatic plasticity effects had compensated for that, confounding the results in this study. Hypothetically, an increase in P_r coupled with a decrease in the presence of postsynaptic receptors could lead to an unaltered input-output curve. Therefore, I decided to use a different approach to analyze possible changes in neurotransmission in the Mover KO: synaptic facilitation. An increase in release probability is usually accompanied by a decrease in facilitation. Hence, I probed the paired-pulse ratio at different inter-stimulus intervals (ISI). This is achieved by applying a pair of stimuli with a variable short interval

between them. Here, the intervals were 20, 40, 80, 150, 300 and 500 ms (Figure 3C). The ratio of the second response to the first response is called the paired-pulse ratio. The ratios, however, did not differ between WT and KO animals (*P*= 0.32 Extra-sum-of-squares F test; *n*=13 each group; *N*=4-5).

Applying a short train of 5 stimuli at 25 Hz led to similar results (Figure 3D): there was no difference between WT and KO fEPSPs (*P*=0.28, two-way ANOVA). Correspondingly, when recording responses to low-frequency stimulation at 0.05 Hz, and then increasing stimulation frequency to 0.2 Hz both groups facilitated to the same degree (Figure 4A; WT 1.4 ± 0.2 n=13 *N*=7; KO 1.2 ± 0.1 *n*=18 *N*=8; P=0.58). Similar results were achieved when further increasing stimulation frequency to 0.5Hz (WT 1.5 ± 0.2; KO 1.4 ± 0.1; P=0.73). Paired-pulse ratios with a 40 ms ISI were also recorded during repeated stimulation at the different frequencies mentioned above (0.05, 0.2 and 0.5 Hz). Ratios also did not vary between WT and KO Schaffer collaterals at 0.05 Hz (Figure 4B; WT 2.15 ± 0.06; KO 2.16 ± 0.10; *P*=0.88), at 0.2 Hz (WT 1.74 ± 0.05; KO 1.73 ± 0.05; *P*=0.92) or 0.5 Hz (WT 1.47 ± 0.03; KO 1.46 ± 0.03; *P*=0.73).

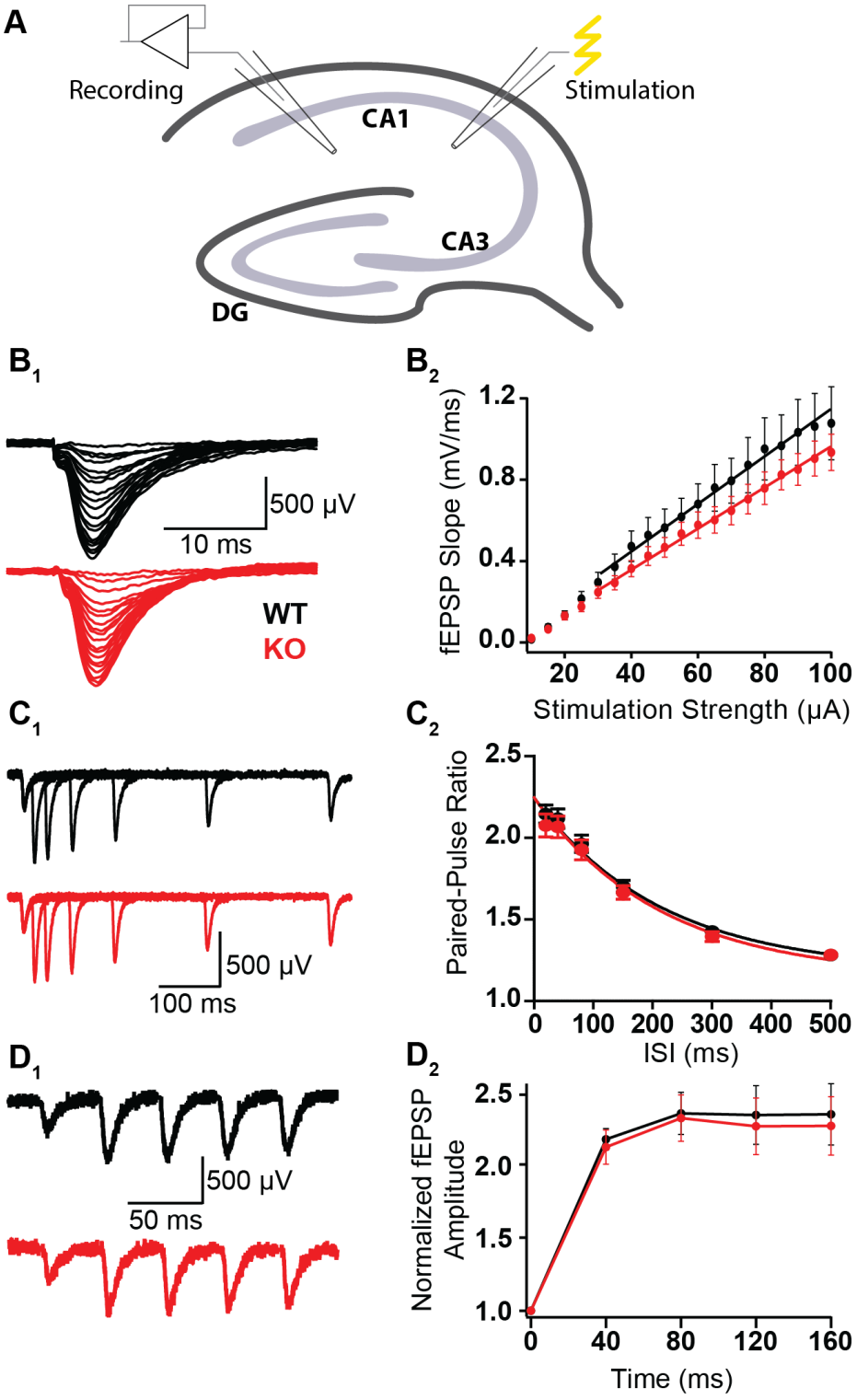

Figure 3: Loss of Mover does not affect transmission at Schaffer collateral synapses.
(A) Diagram representing stimulation of Schaffer collaterals and extracellular recording at stratum radiatum of the CA1. Representative traces of WT (black) and KO (red) fEPSP recordings in response to: increasing stimulation strength (B_1), two stimuli with varying inter-stimulus intervals (C_1) and a train of 5 stimuli at 25 Hz (D_1). (B_2) Input-output curve obtained from responses to increasing stimulation strengths revealed no differences between WT and KO slopes. (*C-D*) fEPSP amplitudes were normalized to that of the first fEPSP. (*C*) Paired-pulse ratios at different inter-stimulus intervals were fit to a mono-exponential function. Fits did not vary between KO and WT. (*D*) Responses to a train of high-frequency (25Hz) stimulation also showed no differences between WT and KO. Error bars represent SEM.

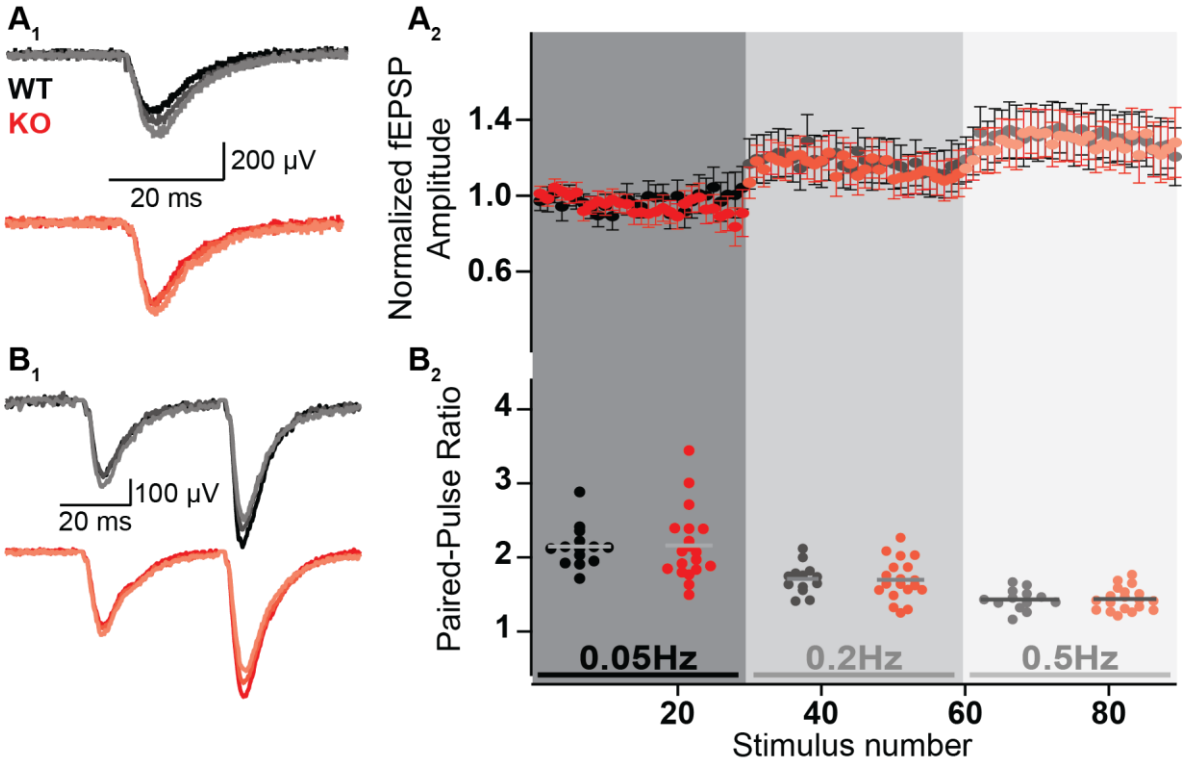

Figure 4: Absence of Mover does not change facilitation at Schaffer collateral synapses. (*A1*, *B1*) Overlaid representative traces of Schaffer collaterals fEPSPs recorded at 0.05 (WT: black traces, KO: red traces), 0.2 (dark grey, light red) and 0.5 Hz (light grey, light pink). (*A2*) Baseline response was recorded at 0.05 Hz for 10 minutes (30 stimuli), followed by an increase in stimulation frequency to 0.2 Hz (light grey background) and another subsequent increase to 0.5 Hz (white background). Amplitudes were normalized to that measured during baseline. Degree of facilitation did not differ between WT and KO for any of the frequencies. (*B2*) Paired-pulse ratios (40 ms ISI) were measured during the different stimulation frequencies. No difference between WT and KO ratios were detected in any of the frequencies measured. Error bars represent SEM.

3.3 Synaptic plasticity is increased at mossy fibers upon Mover knockout

The protein Mover was named after its strong presence in the hippocampal **mo**ssy fibers and because it is **ver**tebrate specific (Kremer et al., 2007). I therefore decided to study the effect of the knockout in this pathway where its absence would arguably have the strongest effect. For such recordings, I stimulated the mossy fibers leaving the dentate gyrus and recorded fEPSPs in the stratum lucidum of the CA3 (Figure 5A).

Similarly to the recordings in the Schaffer collaterals (see above) I studied the effect of the knockout in the input-output relationship in the mossy fibers. Input-output curves were plotted in terms of fEPSP slope versus fiber volley amplitude and fit to a linear regression for each slice, and compared between WT and KO (see section 3.2 of Results above for further details). There were no differences detected between the two groups in the slope of the fit (Figure 5B, WT 0.9 ± 0.1 s^{-1} *n*=18 *N*=9; KO 0.9 ± 0.1 s^{-1} *n*=21 *N*=8; *P*= 0.83) or in the Y-intercept (WT -0.018 ± 0.011 mV/ms *n*=18; KO -0.002 ± 0.007 mV/ms *n*=21; *P*= 0.23). These results were comparable to the lack of change observed in the Schaffer collaterals.

It was interesting to evaluate whether the lack of Mover influenced the exceptional short-term plasticity typically observed in mossy fibers (for a review see Nicoll & Schmitz 2005). Strikingly, when applying electrophysiological protocols that measured synaptic plasticity a strong effect of the knockout of Mover was detected. By applying pair of pulses within a short time interval it was possible to evaluate whether the KO affected the facilitation typically observed at this synapse on a short timescale (Figure 5C). The KO mossy fibers (*n*=13, *N*=4) had a slightly greater paired-pulse ratio than their WT counterparts (*n*=11, *N*=4; *P*<0.05 Extra-sum-of-squares F test).

Using not only pairs of stimuli but repeated stimulation led to an even greater increase in facilitation. The stimulation of the mossy fibers with a short train of 5 stimuli at 25 Hz led to a facilitation of 12-fold in Mover KO (Figure 5D; 12.7 ± 0.6 *n*=22 *N*=7). This represents an increase of almost 50 % over the facilitation in WT mice (8.8 ± 0.8 *n*=18 *N*=6; *P*<0.0001 two-way ANOVA).

Similarly, results with low-frequency stimulation also led to an increased facilitation in the absence of Mover. After recording a baseline response at 0.05 Hz for 10 minutes, the

frequency of stimulation was increased to 0.2 Hz. This increment in frequency is known to increase transmission prominently in the mossy fibers (low-frequency facilitation, for a review see Nicoll & Schmitz 2005; for use of these particular frequencies see e.g. Ben-Simon et al., 2015). As expected, WT mossy fibers responses approximately doubled (Figure 6A; 1.97 ± 0.01 n=18 N=6). Remarkably, transmission in KO mice was increased even more (2.35 ± 0.08 n=22 N=7; P<0.001). The difference was even more evident upon further increasing the frequency to 0.5 Hz with the Mover KO facilitating 40 % more than the WT (WT 3.90 ± 0.26; KO 5.37 ± 0.26; P<0.001). Paired-pulse ratios with an ISI of 40ms during repeated stimulation at 0.05, 0.2 and 0.5 Hz (as described above) were also measured. Ratios were also increased in the KO at 0.05 Hz (Figure 6B, WT 3.01 ± 0.15; KO 3.44 ± 0.14; P<0.05) and at 0.2 Hz (WT 2.23 ± 0.08; KO 2.44 ± 0.06; P<0.05) but the difference becomes too small to be significant at 0.5 Hz (WT 1.65 ± 0.04; KO 1.73 ± 0.04; P=0.17).

These results show by different protocols that there is an increase in facilitation in the mossy fibers in the Mover knockout. They support the idea that the presence of Mover is reducing synaptic plasticity in mossy fiber terminals but not in Schaffer collaterals.

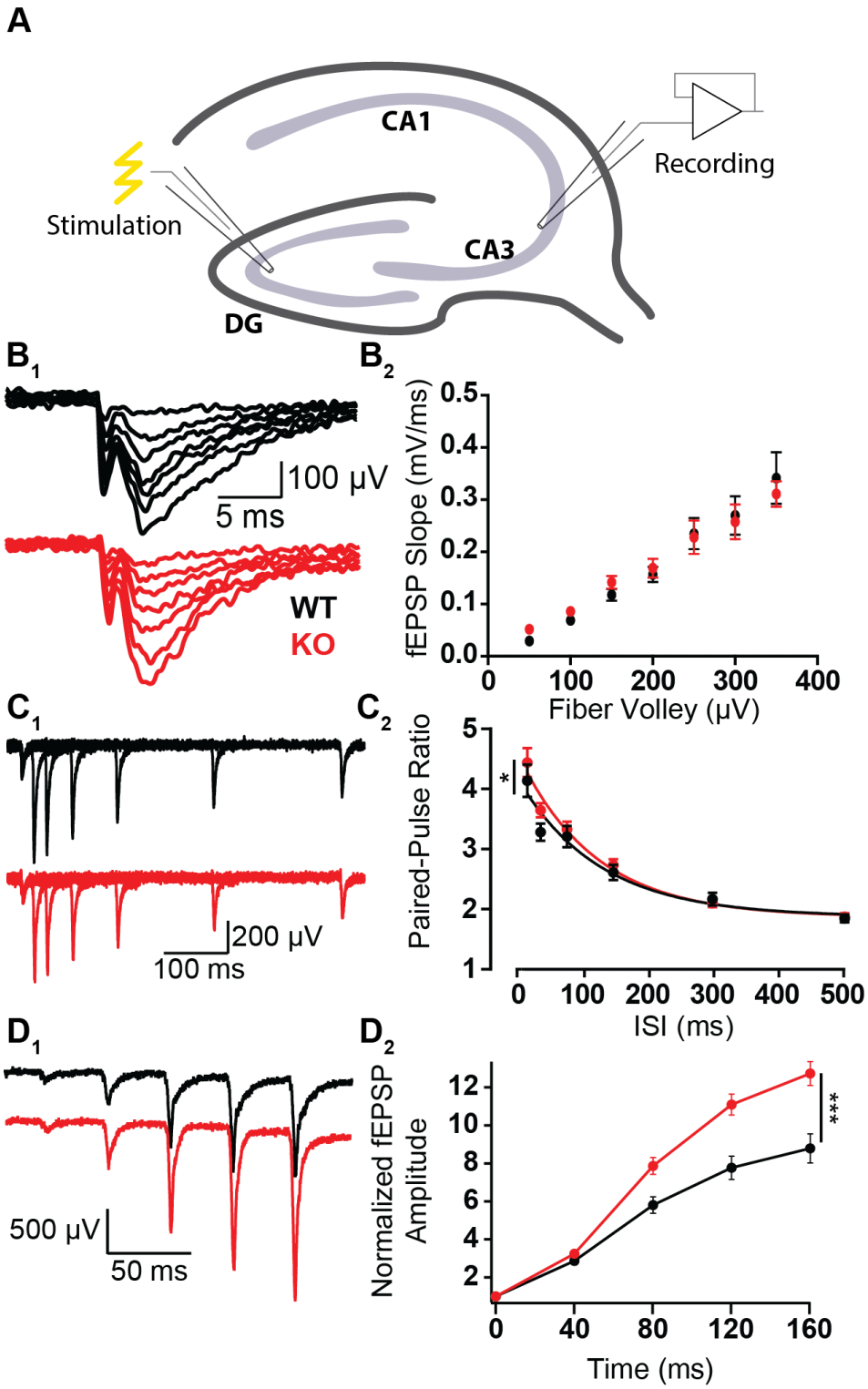

Figure 5: Synaptic facilitation at mossy fiber terminals is increased in the absence of Mover. (*A*) Diagram representing stimulation of mossy fibers and extracellular recording in the stratum lucidum of the CA3. Representative traces of WT (black) and KO (red) fEPSP recordings in response to: increasing stimulation strength (B_1), two stimuli with varying intervals (C_1) and a train of 5 stimuli at 25 Hz (D_1). (B_2) Input-output curve, obtained from responses to increasing stimulation strengths, revealed no differences between WT and KO slopes. (*C-E*) fEPSP amplitudes were normalized to that of the first fEPSP. (*C*) Paired-pulse ratios at different inter-stimulus intervals were fit to a mono-exponential function. KO responses had greater ratios than WT. (*D*) Responses to a train of high-frequency (25 Hz) stimulation also showed increased facilitation in KO mossy fibers when compared to WT. Error bars represent SEM. *:P<0.05; ***:P<0.001

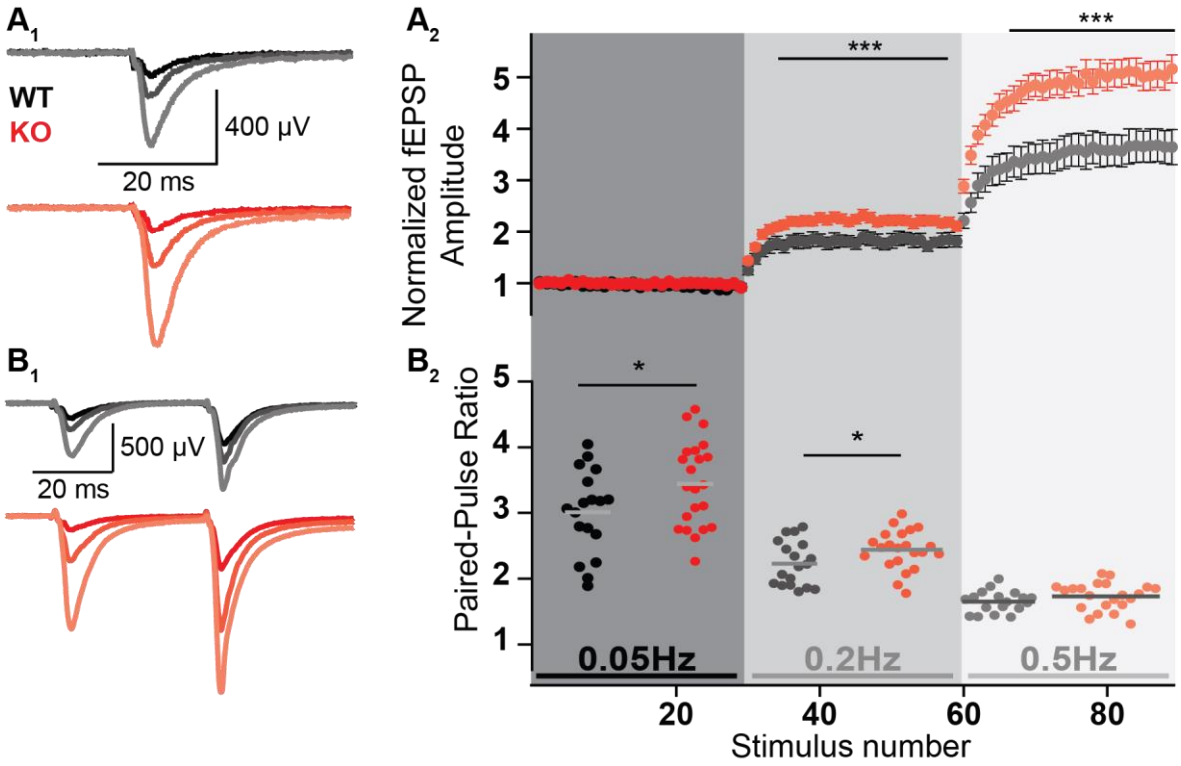

Figure 6: Loss of Mover increases low-frequency facilitation in mossy fibers synapses.
(A_1, B_1) Overlaid representative traces of mossy fiber fEPSPs recorded at 0.05 (WT: black, KO: red), 0.2 (dark grey, light red) and 0.5 Hz (light grey, light pink). (A_2) Baseline response was recorded at 0.05 Hz for 10 minutes (30 stimuli), followed by an increase in stimulation frequency to 0.2 Hz (light grey background) and another subsequent increase to 0.5 Hz (white background). Amplitudes were normalized to that measured during baseline. KO facilitation is strongly increased at both 0.2 and 0.5 Hz. (B_2) Paired-pulse ratios (40 ms ISI) were measured during the different stimulation frequencies. KO mice had increased paired-pulse ratios at 0.05 and 0.2 Hz. Error bars represent SEM. *: $P<0.05$; ***: $P<0.001$

Recordings with stimulation of the mossy fibers can often be confounded by stimulation of associational/commissural fibers. Therefore, the use of a group II metabotropic glutamate receptor agonist ensures that the recorded responses are exclusively from mossy fibers. Such agonists selectively block mossy fiber release and do not affect other synapses in the CA3 (Kamiya et al., 1996). The agonist DCG-IV was applied at the end of mossy fiber recordings to ensure specificity of stimulation (see 'Material and Methods' for details). It systematically blocked responses, ensuring that only mossy fibers were being recruited (Figure 7).

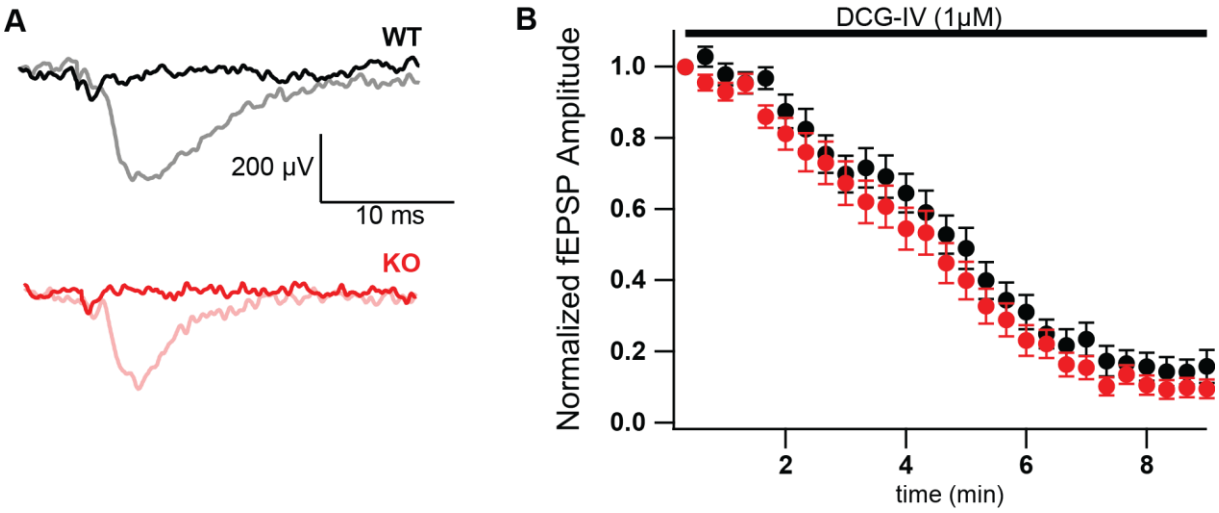

Figure 7: Application of DCG-IV ensured specificity of mossy fiber stimulation.
The group II metabotropic glutamate receptor agonist DCG-IV was used at the end of every experiment with mossy fiber stimulation to test for contamination from other pathways. (*A*) Representative traces of WT and KO responses before (grey and light red, respectively) and after (black/red) application of DCG-IV. (*B*) DCG-IV abolished responses to mossy fiber stimulation. Responses that were not reduced by at least 80 % were not used in the analysis. Error bars represent SEM.

3.4 Increment in Mover KO facilitation is calcium- and age-dependent

Mover has been reported to bind to Calmodulin in a calcium-dependent manner and have a calcium-dependent effect on neurotransmitter release (Körber et al., 2015). It would, therefore, be interesting to test if the difference observed here between WT and KO would also be calcium-dependent.

It is also known that synaptic facilitation in the mossy fibers strongly decreases between the 3rd and 9th week of life in mice (Mori-Kawakami et al., 2003). That led to me to pose the question if it was the presence of Mover that was partially responsible for the reduction in facilitation observed in the development of the CA3. Since I had observed an increase in facilitation in 3 week-old KO mice (Figure 5 and Figure 6) it would appear that Mover is buffering facilitation in the mossy fibers. A hypothetical increase in Mover activity with age could, therefore, explain the reduction in facilitation observed in older mice and I decided to address if that was indeed the case.

To be able to tackle both questions I decided to test the extent of synaptic facilitation in 54-60 days-old (approximately 9 week-old) mice in varying calcium concentrations. Experiments started with an extracellular Ca^{2+} concentration of 1.25 mM, then increased to 2.5 mM and finally to 3.5 mM. The concentration of Mg^{2+} was decreased in each step to keep the concentration of divalent ions constant. A ten-minute waiting period was observed between each change in calcium concentration to allow the new calcium concentration to equilibrate in the solution.

In agreement with the idea that the effect of Mover is calcium-dependent, at 1.25 mM Ca^{2+} the previously observed difference between WT and KO was completely gone (Figure 8A, compare with Figure 5 and Figure 6). When increasing stimulation frequency from 0.05 to 0.2 Hz both WT and KO mossy fibers facilitated to a similar extent (WT 1.7 ± 0.1 *n*=13 *N*=9, KO 1.6 ± 0.1 *n*=13 *N*=9, *P*=0.38). When further increasing the frequency to 0.5 Hz, facilitation still did not differ (WT 4.2 ± 0.5, KO 3.8 ± 0.4, *P*=0.48). Accordingly, responses to a train of 5 stimuli at 25 Hz showed no difference between WT and KO (at last response: WT 13.3 ± 1.7, KO 11.5 ± 1.0: *P*=0.12 two-way ANOVA).

In the same slices, after recording at 1.25 mM Ca^{2+}, the concentration of calcium was increased to 2.5 mM and the same protocols were recorded. This extracellular calcium concentration was the same as in the previously described experiments in 3-week old mice. Surprisingly, in older mice the difference between WT and KO was not present anymore (Figure 8B, compare with Figure 5 and Figure 6). There were no differences in plasticity between WT and KO in frequency facilitation when increasing stimulation frequency from 0.05 to 0.2 Hz (WT 2.0 ± 0.1, KO 2.0 ± 0.1, *P*=0.94) or further increasing it to 0.5 Hz (WT 3.5 ± 0.5, KO 3.3 ± 0.4, *P*=0.81). Also no differences were detected in response to a 25 Hz-train of stimuli (WT 4.6 ± 1.0, KO 4.5 ± 0.7, *P*=0.86 two-way ANOVA).

In my experiments, the difference between 3- and 9-week old WT mice is only observable in the high frequency train of stimuli, in which facilitation reaches 12-fold in 3-week old mice (Figure 5D), and 4.5 in 9-week old mice (Figure $8B_2$), in agreement with previously published results showing a reduction in the amount of facilitation in the mossy fibers of 9-week old mice (Mori-Kawakami et al., 2003). However, in low-frequency stimulation the extent of facilitation is similar in both ages (Figure 6A, Figure $8B_1$). Nevertheless, the absence of difference between WT and KO in 9-week old animals contrasts with the increased facilitation seen in younger animals. This suggests an age-dependency on the effect of Mover. However, this effect seems contrary to what was initially hypothesized in

the beginning of this section. Mover is not the reason why facilitation is decreased in older animals. On the contrary, it seems that Mover activity is downregulated with the decrease of synaptic plasticity. Interestingly, this relates well with the idea of Mover acting as a dynamic buffer in synaptic strength, regulated by activity (see 'Discussion'), since there is stronger inhibition and weaker excitability in older mice (Nurse and Lacaille 1999; Zitman and Richter Levin 2013).

It is important to note that Mover is still present at these synapses in 9-week old WT mice (unpublished data), so that is not the reason for the similar responses between WT and KO in older mice. The results that follow, with 3.5 mM calcium, also corroborate that.

Further increasing the extracellular calcium concentration to 3.5 mM changes the situation. Once again, facilitation in the absence of Mover is increased (Figure 8C). At this high calcium concentration, I observed an increased facilitation in the KO both when increasing stimulation frequency from baseline to 0.2 Hz (WT 1.30 ± 0.03, KO 1.44 ± 0.06, $P<0.05$) and then to 0.5 Hz (WT 1.45 ± 0.06, KO 1.75 ± 0.11, $P<0.05$), as well as in high-frequency train stimulation (WT 1.0 ± 0.07, KO 1.4 ± 0.1: $P<0.0001$ Two-Way ANOVA). This suggests that Mover indeed has a calcium-dependent effect.

When comparing these results with the previous experiments it becomes clear that not only the calcium concentration but also the age of the animals has an influence on the effect of Mover on short-term plasticity. In younger animals the increased facilitation in KO was already present at lower calcium concentrations, denoting a possible calcium- and age-dependent effect of Mover on plasticity. It appears that the effect of Mover on facilitation is shifted to higher calcium concentrations with age in mouse mossy fiber synapses.

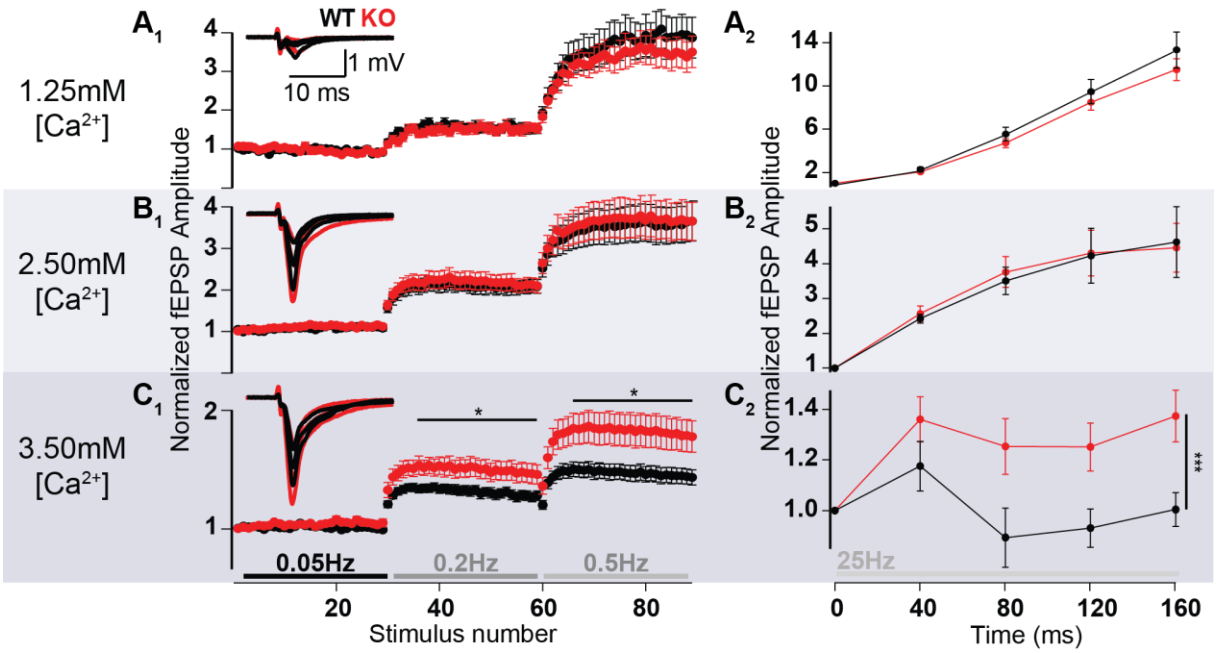

Figure 8: Boost in facilitation in Mover KO is age- and calcium-dependent.
Recordings of fEPSPs in the stratum lucidum upon mossy fiber stimulation of acute hippocampal slices from 54-60 days old mice at three different extracellular calcium concentrations: 1.25 mM *(A)*, 2.5 mM *(B)*, and 3.5 mM *(C)*. *(Insets on top left of each quadrant*) Representative current traces exhibiting frequency facilitation. Mover KO undergoes increased facilitation in the presence of 3.5 mM Ca^{2+} (*C*) but not in 1.25 (*A*) or 2.5 mM (*B*). This contrasts with data from 3-week old mice, where increased facilitation in the KO was observed at 2.5 mM extracellular calcium (Figure 5 and Figure 6). Error bars represent SEM. *: *P*<0.05; ***: *P*<0.001

With such experiments, one can assess not only the effect on short-term plasticity but also on basal synaptic transmission. Raising extracellular calcium concentration promotes an increase in the release of neurotransmitter-laden vesicles (for a review see Neher & Sakaba 2008). The increase in fEPSP amplitudes followed the same proportions in WT and KO (Figure 9). This was calculated by measuring the baseline responses at the three different calcium concentrations. fEPSP amplitudes were then normalized to the responses at 3.5 mM Ca^{2+} and plotted against the extracellular calcium concentration. The dependence between synaptic transmission and extracellular Ca^{2+} concentration was fitted with a power function (Figure 9B) described as:

$$y = Ax^B$$

Where '*B*' describes the power relationship between the increase in extracellular Ca^{2+} and the increase in fEPSP amplitude and '*A*' is a scaling factor (Scott et al., 2008b). As mentioned before, there were no differences in the responses to increasing calcium concentration, which means that the fits did not differ between WT (*B*=2.25 ± 0.23) and

KO (*B*=2.20 ± 0.17; *P*=0.98 Extra-sum-of-squares F test). The power relationship here described corroborates previous findings (Geiger and Jonas, 2000; Scott et al., 2008b).

The lack of change in release would argue against Mover having an effect on calcium-sensitivity of release in the mossy fiber terminals at basal stimulation frequencies. It is possible that Mover is dynamically affecting plasticity, but not strongly interfering with basal release conditions. To evaluate if this effect in plasticity was mediated by the cyclic adenosine monophosphate (cAMP)-pathway, which is known to affect plasticity in the mossy fibers, I decided to use the drug forskolin (see below).

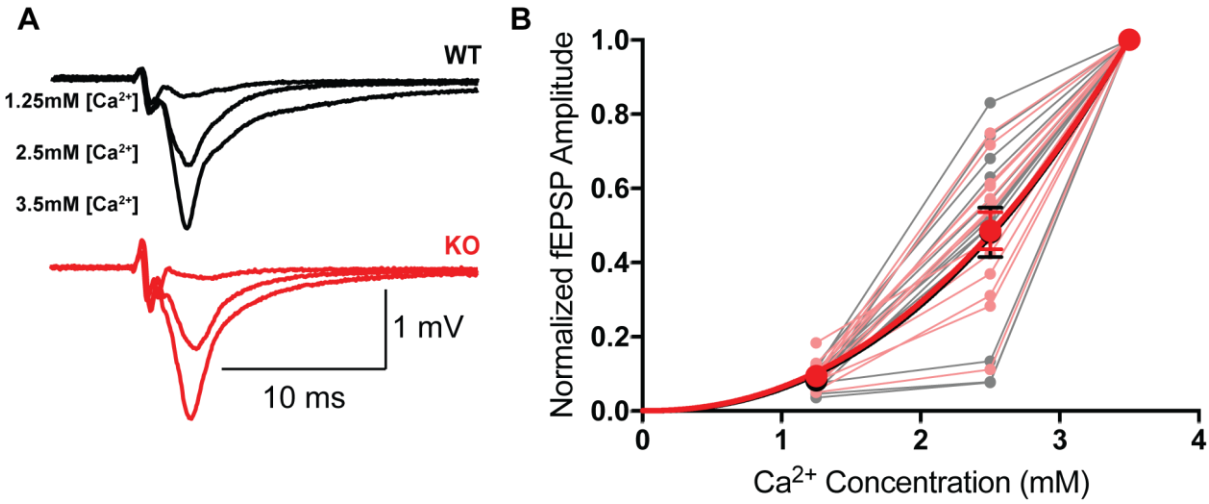

Figure 9: Basal responses increase to a similar extent in WT and KO in response to calcium increase.
(*A*) Overlaid representative traces of WT (black) and KO (red) mossy fiber fEPSPs recorded at baseline stimulation frequency (0.05 Hz) at 1.25, 2.5 and 3.5 mM extracellular Ca^{2+} concentration. (*B*) fEPSP amplitudes were normalized to the response at 3.5 mM Ca^{2+} and fit to a power function. Shaded dots on the background represent each recording of each slice and are connected by lines to the response to different calcium concentration at the same slice. Large dots on the foreground represent the mean value of recordings and curves represent the power function fits. Fits did not vary between WT and KO, revealing no calcium-dependency of the Mover effect on basal release. Error bars represent SEM.

3.5 Forskolin occludes increase in facilitation from KO

Forskolin is an Adenylyl Cyclase (AC) activator, which has been shown to affect plasticity at the mossy fibers but have no effect on the associational/commissural fibers (Weisskopf et al., 1994). Namely, it robustly induces long-lasting potentiation of glutamate release at the mossy fibers. Conversely, inhibiting the Protein Kinase A (PKA) pathway in the presynapse, which is downstream of AC (see Figure I.7), leads to a blockade of long-term potentiation in mossy fiber synapses but not in the CA1 (Weisskopf et al., 1994). Hence, manipulations of either Mover or the PKA pathway revealed similar results: synaptic plasticity changes in the mossy fiber but not in the Schaffer collateral. The similarity between these results made the activation of the PKA pathway through forskolin particularly interesting to investigate in the knockout. With this tool I could assess both the effect of the lack of Mover on long-term plasticity, which is elicited by forskolin, but also whether Mover and PKA participate in the same pathway.

Application of 10 µM forskolin for 10 min led to a long-lasting potentiation in synaptic transmission of around 2.4-fold in both WT (Figure 10A; 2.38 ± 0.15 n=10 N=3) and KO (2.45 ± 0.11 n=10 N=3; P=0.73). This lack of change in cAMP/PKA-dependent effect suggests that Mover is not involved in evoking long-term potentiation in these synapses, at least not downstream of AC in this pathway. We cannot rule out its participation upstream of AC or in other forms of long-term potentiation (LTP) that have been described at this synapse, e.g. Protein Kinase C-dependent postsynaptic recruitment of NMDA receptors (Kwon and Castillo, 2008).

After application of forskolin, the facilitation of neurotransmitter release was also tested. Strikingly, the increased facilitation previously observed in the KO (Figure 5 and Figure 6) was eliminated after potentiation by forskolin (Figure 10). When increasing the frequency of stimulation from 0.05 Hz to 0.2 Hz, both WT and KO fEPSPs facilitated to similar levels (Figure 10A; WT 3.92 ± 0.25; KO 3.53 ± 0.23; P=0.27). Furthermore, after increasing stimulation frequency to 0.5 Hz, there was no difference in the facilitation between WT (6.99 ± 0.40) and KO (KO 6.44 ± 0.49; P=0.40). Correspondingly, facilitation was similar between WT (Figure 10B; 7.2 ± 0.7 at last response) and KO (7.6 ± 0.7; P=0.54 two-way ANOVA) in response to a 25 Hz train of stimuli, whereas in the absence of forskolin-induced potentiation, I had observed an increase of facilitation 50 % bigger in the KO (Figure 5D).

Activation of the cAMP/PKA pathway by forskolin has, therefore, occluded the effect that the knockout had on synaptic facilitation. This suggests that Mover could act in the same pathway as forskolin. It is arguable that Mover would act upstream of the activation of AC. Thus, the use of forskolin would bypass the absence of Mover by directly activating AC and increasing facilitation (also see Figure D.1).

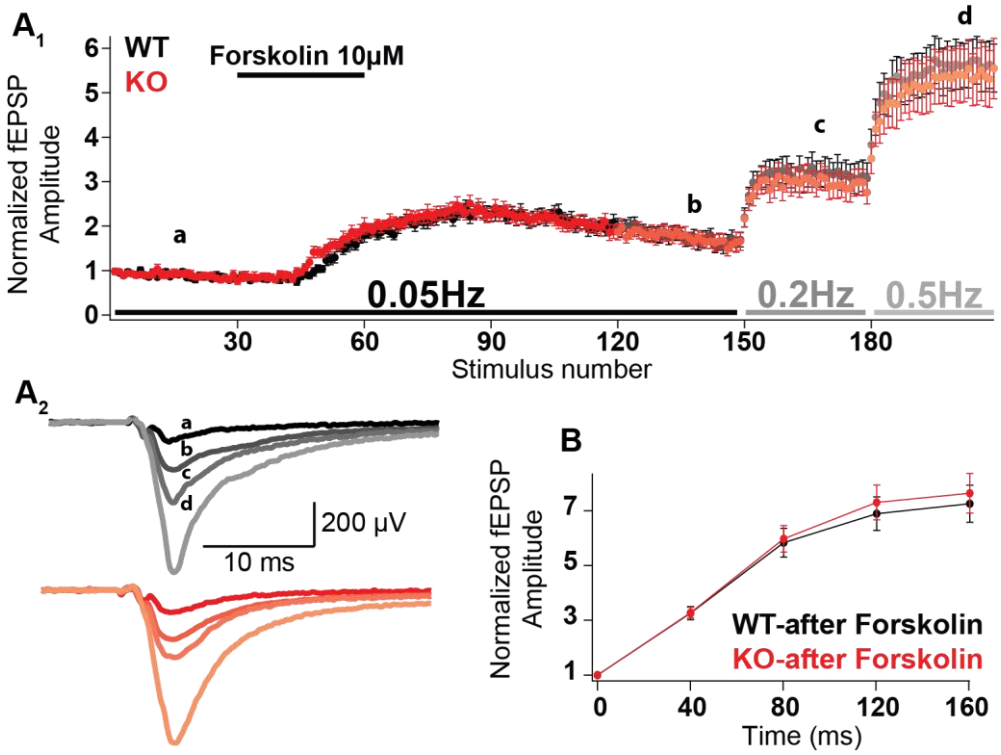

Figure 10: Mover KO does not affect forskolin-induced potentiation but potentiation restores KO facilitation back to WT levels.
(*A₁*) Normalized amplitudes displayed during the time course of the experiment in which forskolin (10 µM) is applied for 10 minutes to WT (black/grey) and KO (red/orange). During the recording of the baseline (*a*), application of forskolin and after washout (*b*), the stimulation is performed at 0.05 Hz. Afterwards stimulation frequency is increased to 0.2 (*c*) and 0.5 Hz (*d*). At every point, the extent of facilitation was similar between WT and KO. Amplitudes were normalized to amplitudes recorded at baseline (*a*). (*A₂*)Representative traces corresponding to responses before (*a*) and after (*b*) Forskolin application and after increasing stimulation frequency to 0.02 (*c*) and 0.5 Hz (*d*). (*B*) Responses to a train of high-frequency stimulation after forskolin-induced potentiation also showed no differences between WT and KO. fEPSP amplitudes of the train were normalized to the amplitude of the response to the first stimulus of the train. Error bars represent SEM.

3.6 No change in miniature EPSCs in CA3 pyramidal cells in the absence of Mover

To better dissect the effect of Mover on mossy fiber transmission I switched from extracellular to intracellular recordings. Voltage-clamp whole-cell recordings from CA3 pyramidal cells were performed for the recording of postsynaptic currents. As an initial approach, to ensure that there was no change in quantal properties of synaptic transmission, I recorded miniature EPSCs from these cells in acute hippocampal slices. This was done in the presence of 1 µM TTX, similar to what I have described for dissociated cultures (see section 3.1 of Results), and also in the presence of the GABA receptor blocker picrotoxin (100 µM).

As expected, the analysis of spontaneous synaptic transmission in CA3 pyramidal cells did not reveal any differences between WT and KO mEPSCs (Figure 11). The amplitudes were unchanged both when comparing the means of each cell (Figure 11C_1, WT 32.0 ± 1.9 *n*=15 *N*=6; KO 33.3 ± 2.9, n=11 *N*=5; *P*=0.66), and when comparing the cumulative distribution of the amplitudes (Figure 11C_2; *P*=0.30, Kolmogorov-Smirnov test). The frequency (WT 1.63 ± 0.35 Hz; KO 2.86 ± 0.97; *P*=0.20), rise time (10-90 % rise time: WT 1.82 ± 0.12 ms; KO 1.73 ± 0.21 ms; *P*=0.68) and decay (Tau: WT 11.24 ± 0.45; KO 10.74 ± 1.19; *P*=0.66) also did not differ between WT and KO (Figure 11D-F).

Even though changes in mEPSCs in the CA3 pyramidal cells were not expected, this was an important starting point for the next experiments: whole-cell recordings with stimulation of the mossy fibers.

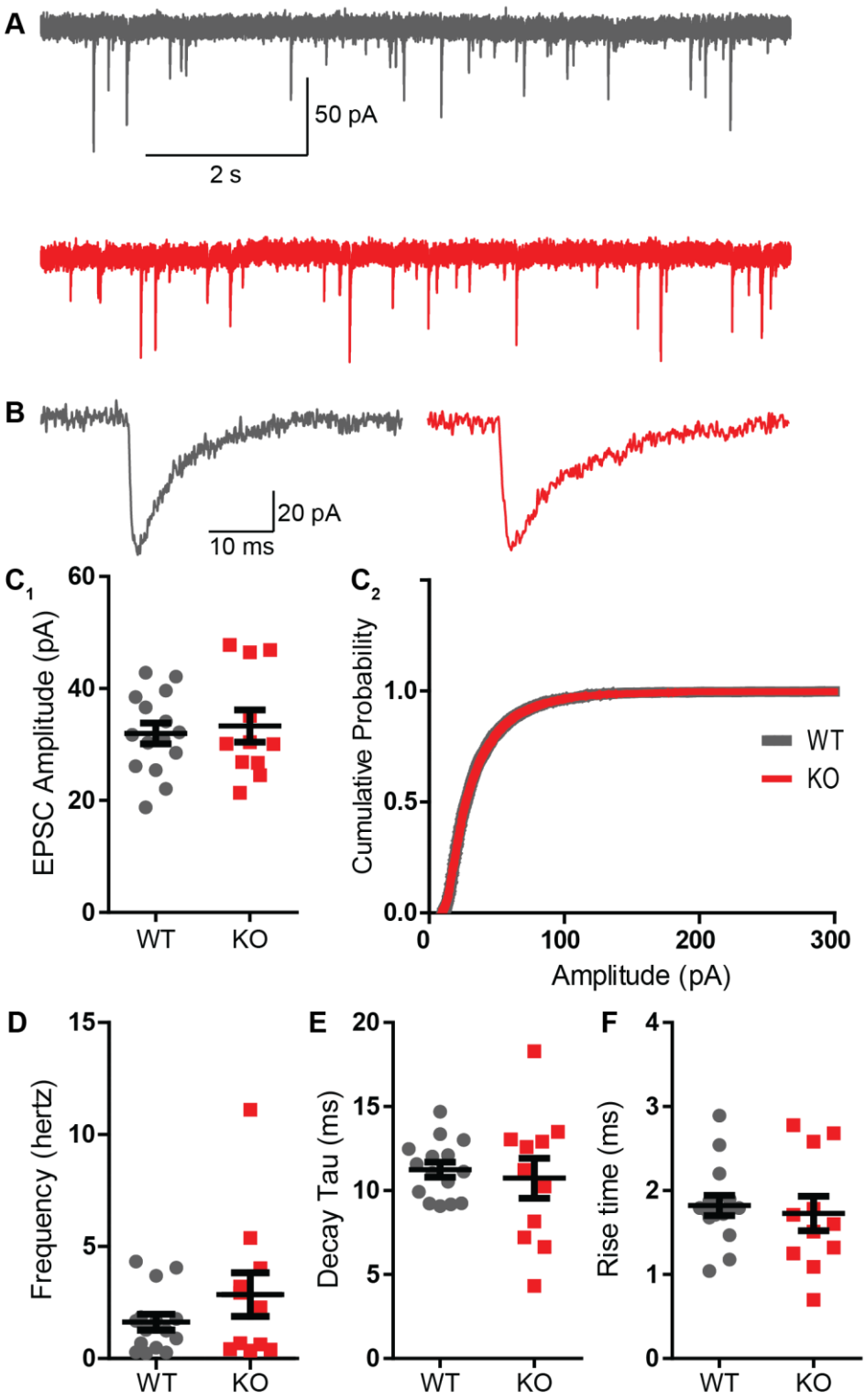

Figure 11: No changes in CA3 pyramidal cell mEPSCs upon knockout of Mover.
Whole cell recordings of miniature EPSCs in CA3 pyramidal cells. (*A-B*) Representative current traces of mEPSC recordings in WT and KO pyramidal cells. (*B*) Representative mEPSC event. (*C*) mEPSC amplitudes show no difference in terms of means of each cell (C_1) nor when comparing their cumulative distribution (C_2). (*D*) Frequency, (*E*) 10-90 rise time, and (*F*) time constant of decay are unchanged. Error bars represent SEM.

3.7 NMDA-EPSCs reveal increased paired-pulse ratio in mossy fiber transmission, but not in frequency facilitation

To better dissect the effects of Mover knockout in mossy fiber transmission I recorded responses from CA3 pyramidal cells in response to mossy fiber stimulation. These experiments were performed in the presence of the AMPA/Kainate receptor antagonist NBQX (10 µM), and the GABA receptor antagonist picrotoxin (100 µM). The use of picrotoxin here allows for a pure mossy-fiber to pyramidal cell response without the influence of inhibitory transmission. This is often not possible because the high number of reciprocal connections in the hippocampus can transform a small stimulus into strong epileptiform reverberating activity when GABA transmission is blocked (Hablitz, 1984; Traub et al., 1993). However, blocking AMPA conductance by NBQX prevents such epileptiform activity. The use of NBQX has further purposes in this study. One major reason for the use of such a drug is to limit recurrent CA3 network activity. The stimulation of a mossy fiber axon could lead to the spiking of a pyramidal cell, which, in its turn, stimulates the cell that is being recorded. This would lead to recordings that are contaminated by synaptic transmission from associational fibers. However, with AMPA transmission blocked by NBQX and NMDA transmission blocked by Mg^{2+} in non-depolarized cells it is possible to record clean responses in the cell which is voltage-clamped at 30 mV. These are, therefore, NMDA-EPSCs (see more on NMDA receptors in section 1.2.2 ‘Ion Channels and ionic currents’).

The blockade of AMPA transmission to record NMDA-EPSCs in the CA3 is not exclusive to this study, having being used numerous times (e.g. Salin et al., 1996; Kaeser-Woo et al. 2013; Jackman et al. 2016). Still, there was a reason for the use of NBQX, which goes beyond preventing recurrent excitation. This drug blocks not only AMPA receptors but also Kainate receptors (Sheardown et al., 1990). Kainate receptors are known to participate in facilitation of mossy fiber transmission (Lauri et al., 2001b; Schmitz et al., 2001; Breustedt and Schmitz, 2004; but see also Vignes et al., 1998; Kamiya and Ozawa, 2000; Schmitz et al., 2000, which describe an inhibitory effect of kainate receptor activation). Binding of glutamate to Kainate receptors increases neurotransmitter release through the activation of the cAMP/PKA pathway (Rodríguez-Moreno and Sihra, 2004).

The experiments I have described using forskolin have already suggested an effect of Mover in this pathway, where activation of Adenylyl Cyclase led to reversion of facilitation in KO back to WT levels (Figure 10). Therefore, it would be interesting to check whether the difference between WT and KO would disappear, in case this pathway is blocked by NBQX. That was indeed the case, at least partially. As expected, facilitation did not differ between WT and KO in response to a train of stimuli at 25 Hz (Figure 12C; in last EPSC: WT 10.6 ± 1.5; KO 10,6 ± 1.0; P=0.50 two-way ANOVA) or to low-frequency stimulation (Figure 12D; *0.2 Hz*: WT 1.85 ± 0.12 n=10 N=5, KO 1.81 ± 0.12 n=11 N=5, P=0.81; *0.5 Hz*: WT 3.63 ± 0.25, KO 3.73 ± 0.39, P=0.84). Surprisingly, the paired-pulse ratio still showed an increased facilitation in Mover KO mossy fibers (Figure 12B; WT n=9 N=5; KO n=10 N=5; P<0.01 Extra-sum-of-squares F test).

The lack of change in low- and high-frequency facilitation, similar to what was observed upon forskolin application (Figure 10), suggests that Mover indeed participates in the pathway downstream of the activation of the Kainate receptors. However, the increased paired-pulse ratio upon Kainate receptor blockade suggests that Mover's mechanism of action is not exclusive to this receptor's pathway.

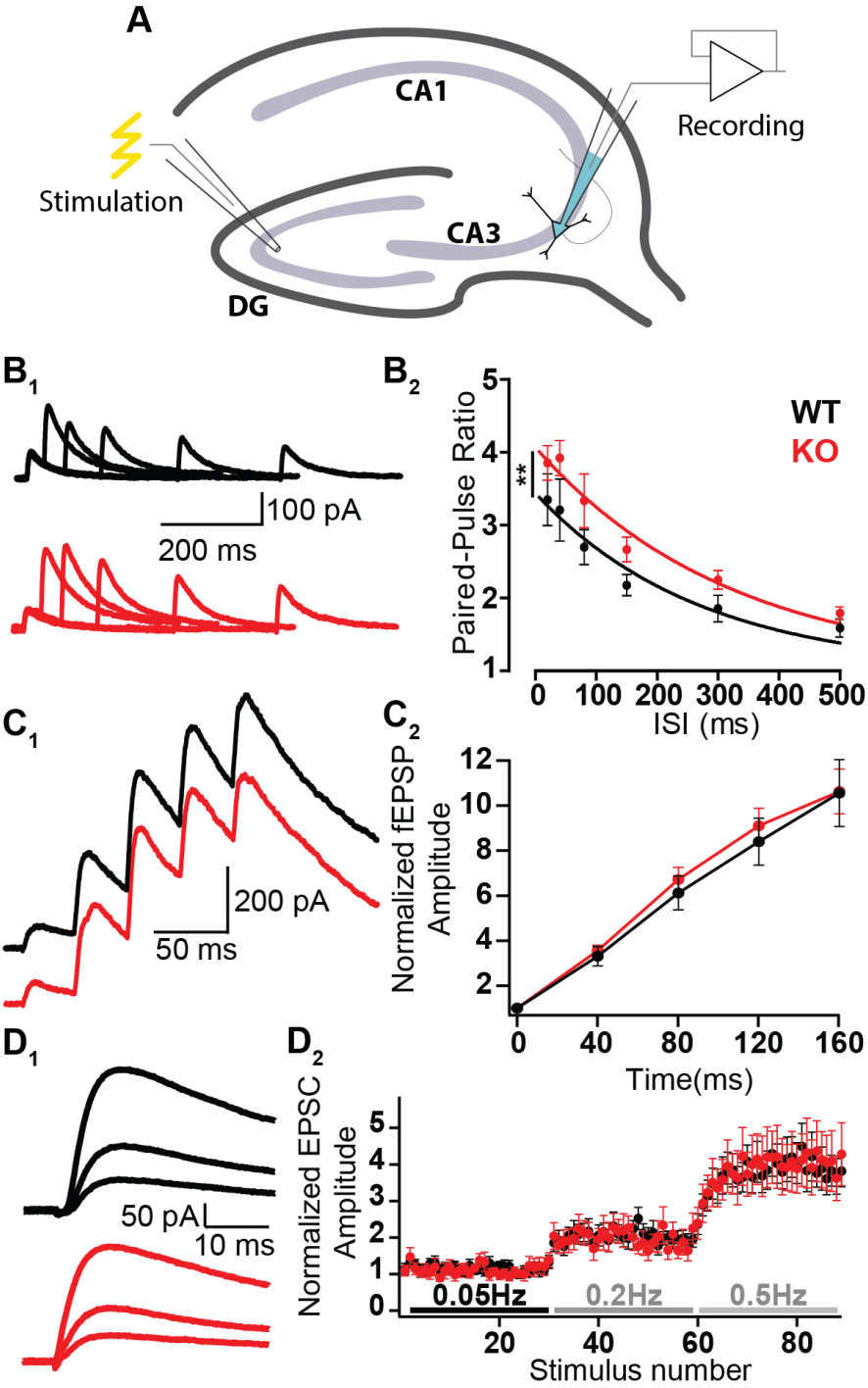

Figure 12: Mossy fiber-evoked NMDA-EPSCs in KO show increased paired-pulse ratio but unchanged frequency facilitation.

(*A*) Diagram representing stimulation of mossy fibers and whole-cell patch-clamp recording of CA3 pyramidal cell. Representative traces of WT (black) and KO (red) EPSCs in response to two stimuli with varying inter-stimulus intervals *(B_1),* to a train of 5 stimuli at 25 Hz (*C_1*) and to stimulation at different frequencies (D_1). (*B-C*) EPSC amplitudes were normalized to the first EPSC amplitude. (*B*) Paired-pulse ratios at different inter-stimulus intervals were fit to a mono-exponential decay. KO responses had greater ratios than WT. (*C*) Responses to a train of high-frequency stimulation showed no difference in facilitation in KO mossy fibers when compared to WT. (*D*) Baseline responses were recorded at 0.05 Hz for 10 minutes (30 stimuli), followed by an increase in stimulation frequency to 0.2 Hz and another subsequent increase to 0.5 Hz. Amplitudes were normalized to the baseline amplitude. Facilitation did not vary between WT and KO. Error bars represent SEM. **: *P*<0.01.

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4 DISCUSSION

This thesis describes for the first time the effect of Mover knockout on synaptic transmission in the hippocampus. Electrophysiological approaches employing extra- and intracellular recordings show increased facilitation in hippocampal mossy fibers to CA3 pyramidal cells of KO mice. Both paired-pulse ratio and responses to low- and high-frequency stimulation showed stronger facilitation in mossy fiber synapses. However, input-output relationship of responses was unchanged.

Activation of the cAMP pathway by forskolin led to similar long-term potentiation of responses in WT and KO mossy fiber synapses. However, this potentiation occluded the increased facilitation previously observed in the KO. I observed a similar occlusion by blocking Kainate receptors. However, in this case, paired-pulse ratios remained increased in the KO.

The increased synaptic facilitation in the absence of Mover was more prominent in younger mice. In older mice, stronger facilitation in KO in comparison with WT responses was only observed at higher calcium concentrations. Finally, the absence of Mover did not affect responses in the downstream synapse: Schaffer collaterals to CA1 pyramidal cells. Nor did it affect the quantal properties of single vesicle release (mEPSC) in mossy fiber to CA3 pyramidal cell or in hippocampal neuronal cultures.

These results indicate that Mover negatively regulates synaptic plasticity in the mossy fiber synapses in an age- and calcium-dependent manner. Also, it has no effect on basal release properties of this synapse, or on the short-term plasticity of Schaffer collateral synapses. Furthermore, the interaction between the effects observed in the KO and their occlusion by

forskolin or by blockade of Kainate receptors suggest that Mover participates in the cAMP pathway. I discuss below a possible model (Figure D.1) that fits these results, as well as the absence of change observed in Schaffer collaterals and previous data from the knockdown of Mover in the calyx of Held (Körber et al., 2015). These results suggest that Mover dynamically buffers synaptic strength and that could have implications to processes such as superpriming and the synaptic computation, as well as to diseases such as schizophrenia and epilepsy, which will be discussed below.

Age-dependent effect and activity-dependent expression of Mover

This study shows that the presence of Mover reduces synaptic facilitation in the mossy fibers. One curious discovery in this project was the age-dependency of the Mover effect. It is known that facilitation in the mossy fibers decreases from juvenile to young adult mice (Mori-Kawakami et al., 2003). In Mori-Kawakami et al. (2003) it is suggested that the developmental change in facilitation is due to a change in calcium sensitivity of release, in particular in the facilitation site (recently suggested to be Synaptotagmin 7, see Jackman et al., 2016). Since Mover has also been suggested to reduce calcium sensitivity of release (Körber et al., 2015), I speculated that Mover could participate in this reduction in facilitation. If that was the case, the difference between the facilitation in WT versus KO would be even greater in older animals than it was in younger ones. Surprisingly, the result was the contrary of what was expected: KO synapses showed almost no difference to WT synapses in older animals. Thus, the effect of Mover on facilitation in fact weakened with development.

The reduction of Mover activity with age, however, correlates well with the idea that Mover is a dynamic regulator of synaptic plasticity, whose expression is regulated by activity. Mover has been shown to be downregulated with the blockade of activity by TTX or sensory deprivation (Kremer, 2008; Wetzel, 2015) and upregulated upon stimulation of activity by forskolin (Moritz Arndt, personal communication). *In vivo*, Otoferlin knockout mice have reduced activity in the auditory pathway due to a lack of neurotransmitter release in the inner hear cells (Roux et al., 2006) and have also reduced Mover presence in the subsequent synapse (Wetzel, 2015). This suggests that a decrease in activity leads to decreased Mover levels. Further evidence suggests that there is a decrease in activity in the hippocampus of mice from the juvenile age to young adulthood: inhibition develops further (Nurse and Lacaille, 1999) and excitability decreases (Zitman and Richter-Levin, 2013).

Since the hippocampus is also greatly involved in the formation of new memories, it is also possible to speculate that the conditions in which lab mice are housed do not offer a stimulating environment for hippocampal activity. This possible reduction in hippocampal activity would correlate well with weaker Mover activity in the hippocampus. However, it is not yet known if the expression of Mover is weaker in the mossy fiber terminals of adult versus young mice. This will be subject of future studies. The idea of a dynamic expression of Mover in response to neuronal activity will be further developed in section 4.2.2 'Mover dynamically buffers synaptic strength'.

4.1 Hypotheses for Mover's Mechanism of Action

4.1.1 Mover participates in the Kainate Receptor/cAMP pathway

Elevation of cAMP levels in the mossy fibers is known to potentiate release at the mossy fibers (Hopkins and Johnston, 1988; Huang et al., 1994; Weisskopf et al., 1994). The activation of Adenylyl Cyclase (AC) by forskolin and subsequent synthesis of cAMP increases release in the mossy fibers whereas the release at Schaffer collateral synapses remains unchanged (Weisskopf et al., 1994; Lonart et al., 1998), and such application of forskolin has been widely used in the field (Bortolotto et al., 1999; Lauri et al., 2001a; Rodríguez-Moreno and Sihra, 2004; Lanore et al., 2010; Ben-Simon et al., 2015). Accordingly, this increase in glutamate release involves the cAMP-regulated PKA (Rodríguez-Moreno and Sihra, 2004). Group II mGluRs also participate in this pathway by inhibiting cAMP synthesis. Activation of group II mGluRs by 1 Hz stimulation leads to long-term depression (Tzounopoulos et al., 1998), suggesting that the connection between cAMP and release strength is quite strong. Furthermore, treatment with the agonist DCG-IV leads to complete arrest of vesicle exocytosis, partially by decreasing cAMP concentration (Kamiya et al., 1996; Kamiya and Yamamoto, 1997; Maccaferri et al., 1998).

Kainate receptors are also known to have a strong effect on mossy fiber transmission. Activation of these presynaptic receptors increases synaptic release probability, suggesting a role of these receptors in the mechanism of activity-dependent facilitation (Lauri et al., 2001a, 2001b; Schmitz et al., 2001; Ji and Stäubli, 2002; Rodríguez-Moreno and Sihra, 2004). This Kainate receptor-dependent synaptic enhancement is also linked to the cAMP

pathway as they occlude each other (Lauri et al., 2001a; Rodríguez-Moreno and Sihra, 2004), and inhibition of PKA suppresses the kainate-induced synaptic facilitation (Rodríguez-Moreno and Sihra, 2004).

Some of the results presented here argue in favor of the participation of Mover in this pathway, specifically inhibiting it. The activation of AC by forskolin occludes the effect that the knockout of Mover had on facilitation, suggesting that it bypasses the inhibition by Mover. Furthermore, blocking Kainate receptors by NBQX also occludes the difference between WT and KO responses to high- and low-frequency stimulation. The blockade of the pathway by NBQX overshadows the inhibition by Mover, masking differences between WT and KO. These results suggest that Mover would be acting in this pathway, between the activation of Kainate receptors and the activation of AC. I will, therefore, elaborate on the model I have developed based on these results and on the literature in the field (Figure D.1).

Model of proposed Mover participation in the cAMP pathway in the mossy fiber synapse

Synaptic activation of Kainate receptors can lead to an increase in synaptic calcium that ultimately leads to increased neurotransmitter release through the AC pathway (Kamiya et al., 2002; Lauri et al., 2003; Scott et al., 2008a; Andrade-Talavera et al., 2012). This calcium increase has been described to be a facilitation of Ca^{2+} entry through VGCCs (Kamiya et al., 2002), through mobilization of Ca^{2+} stores (Lauri et al., 2003; Scott et al., 2008a) or through direct permeation through the Kainate receptor (Lauri et al., 2003; Andrade-Talavera et al., 2012). Importantly, a single stimulation of a single mossy fiber has been described to be sufficient to lead to this increased intracellular Ca^{2+} concentration (Scott et al., 2008a). The rise in calcium concentration stimulates AC activity due to increased Ca^{2+}/CaM binding (Rodríguez-Moreno and Sihra, 2004; Andrade-Talavera et al., 2012). In the mossy fibers, there are two described isoforms of AC that are sensitive to Ca^{2+}/CaM: AC1 and AC8 (for a review on the interplay between calcium and ACs see Halls & Cooper, 2011). Importantly, AC1 is known to have a higher affinity to Ca^{2+} but there has been some evidence that it is not directly present in the presynaptic terminal of cultured neurons, whereas AC8 has a lower Ca^{2+} affinity but is present at the synapse (Nielsen et al., 1996; Wang et al., 2003). In addition, deletion of either AC1, AC8, or both isoforms, impairs plasticity in the mossy fibers (Villacres et al., 1998; Wang et al., 2003).

Deletion of AC1 weakens LTP but does not affect STP (Villacres et al., 1998), whereas deletion of AC8 affects both LTP and STP (Wang et al., 2003). However, neither LTP nor STP was completely abolished in the AC1/8 double knockout, indicating that this pathway is not the only one that leads to enhancement of release (Wang et al., 2003).

Here is where Mover could be playing its part (see Figure D.1). This study shows that Mover knockout has increased synaptic facilitation of release in the mossy fiber synapse. Furthermore, blocking kainate receptors occludes the KO increase in facilitation. As described above, it is known that activation of AC by Ca^{2+}/CaM leads to increased facilitation. Mover has also been shown to bind to CaM in a Ca^{2+}-dependent manner (Körber et al., 2015). Therefore, Mover inhibiting the CaM-AC interaction would lead to weaker facilitation, and this effect would be Ca^{2+}-dependent. In the KO, the inhibition would be lifted and the synapse would, therefore, show an increased facilitation, which is exactly the result described in this study (Figure 5 and Figure 6), and the effect of Mover was indeed Ca^{2+}-dependent (Figure 8). Additionally, direct activation of AC by forskolin would bypass the Mover inhibition and, hence, Mover's presence or absence would make no difference to facilitation. This was also observed in this study (Figure 10). Lastly, Mover absence would not have a strong effect on facilitation in a presynaptic terminal where the AC pathway does not play a strong role in regulating facilitation of release. This is the case at the Schaffer collaterals (Weisskopf et al., 1994; Lonart et al., 1998) and is in agreement with my results (Figure 3).

The only result that is not readily explained by this model is that the paired-pulse ratio differs between WT and KO when Kainate receptors are blocked. However, as previously described, activation of AC is not the only mechanism leading to facilitation at this synapse and it is possible that Mover is involved in other pathways as well. Since CaM participates in many pathways it is possible to expect that Mover could also affect CaM binding to other proteins. This will be subject of future studies.

The cascade of events that follow activation of AC and lead to changes in release involves the synthesis of cAMP by AC and activation of PKA by cAMP (Weisskopf et al., 1994; Tzounopoulos et al., 1998). PKA then activates RIM1α (Rab-interacting molecule 1α), which, together with its interaction partner Rab3A, leads to increased release (Castillo et al., 1997, 2002; Lonart et al., 1998). Notably, these proteins were implicated in regulating release in the mossy fibers in LTP, but not in STP. A different cAMP-activated protein has

also been implicated in influencing the plasticity of mossy fiber terminals, namely Epac2[1] (exchange protein directly activated by cAMP 2; Fernandes et al., 2015). In agreement with the lack of changes observed here in the Schaffer collaterals, Epac2 knockout did not affect responses from axons of CA3 pyramidal neurons (Fernandes et al., 2015). Curiously, at cerebrocortical nerve terminals, Epacs have been shown to indirectly enhance RIM1α-Rab3A interaction (Ferrero et al., 2013). The presence of Rab3A in this pathway is particularly interesting, since both Rab3A and Mover have been implicated in a phenomenon called superpriming (Schlüter et al., 2006; Körber and Kuner, 2016). This will be further discussed in section 4.1.3.2 'Mover, Rab3 and Superpriming'.

The participation of Mover in the inhibition of this pathway is further supported by the idea that cAMP levels at this synapse are tightly regulated. Another important inhibitory factor of AC activity is mGluR2 (Tzounopoulos et al., 1998).

[1] Fernandes et al. 2015 declares that knockout of Epac2 does not affect facilitation during short trains. However, they also show responses to a 10 Hz train in which there are obvious differences (Fig. 7A). It is also the experiment from which they derive the conclusion of the change in RRP.

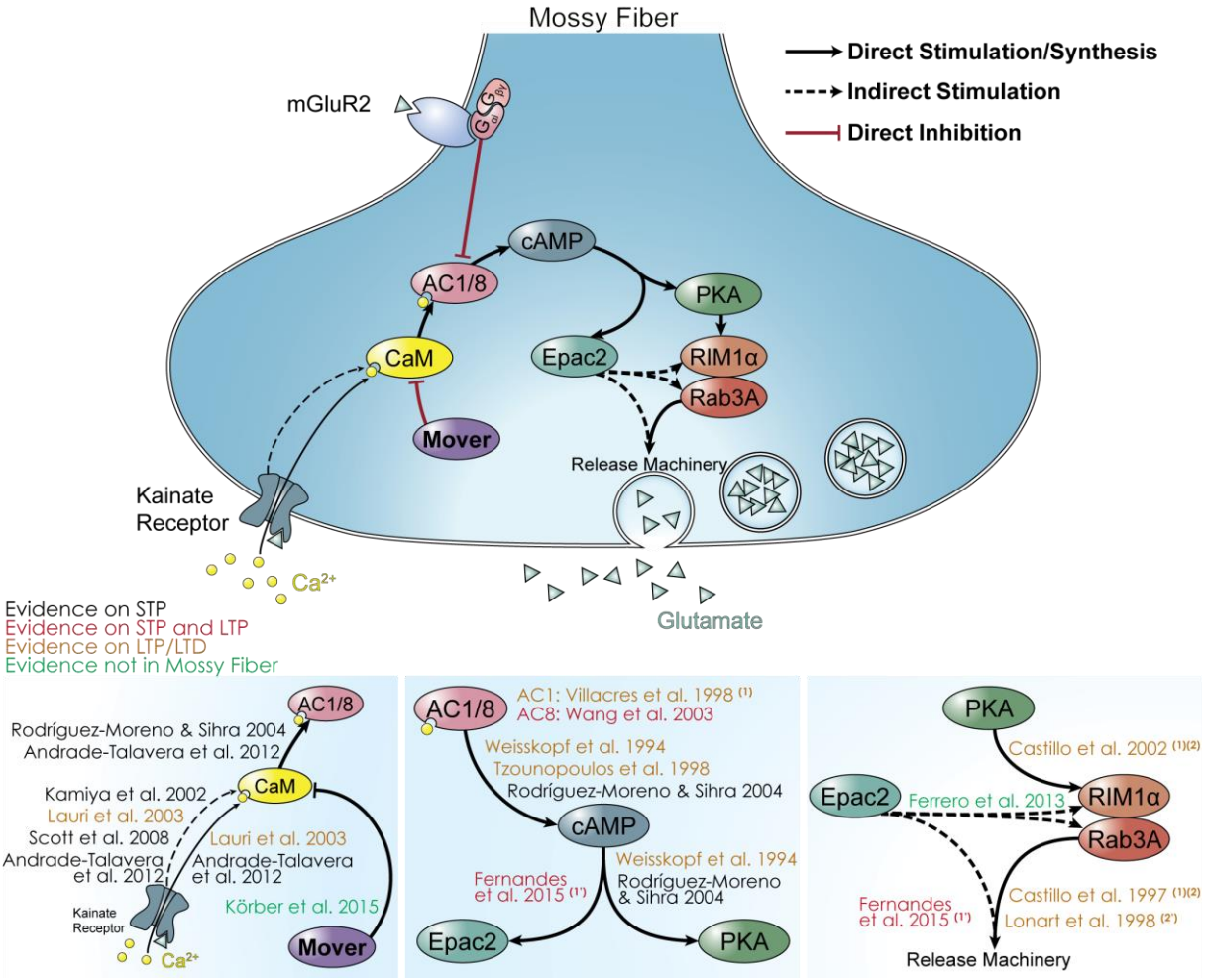

Figure D.1: Schematic model of the proposed action of Mover in the Adenylyl Cyclase pathway of facilitation of release in the hippocampal mossy fiber synapse.

(*Top*) General scheme of the proposed Mover action in the mossy fiber terminal. The binding of glutamate to the Kainate receptor leads to the opening of the channel, which is permeable to calcium. Ca^{2+}/CaM stimulates AC1 and/or AC8 and leads to the synthesis of cAMP. cAMP activates PKA, which can bind to RIM1α, which, together with Rab3A, can lead to increase of neurotransmitter release. cAMP can also directly activate Epac2, which indirectly stimulate RIM1α/Rab3A interaction. Activation of mGlur2 can lead to G-protein inhibition of AC. Mover is proposed to inhibit the pathway by binding to CaM, therefore reducing cAMP synthesis and, ultimately, diminishing the facilitation of release. See text for full description. Position of molecules does not necessarily reflect their position at the presynaptic terminal.

(*Bottom*) Insets of parts of the model with relevant references to specific molecules in the pathway. References describe the participation of the molecule or interaction between molecules in STP (*black*), in STP and LTP (*red*), and in LTP or LTD (*yellow*) in the mossy fiber. References in green describe interactions between proteins that have not been tested specifically in this pathway at the mossy fiber synapse. (*1*) STP tested and unchanged. (*1'*) Paired-pulse ratio unchanged, but response to a train of stimuli affected. (*2*) Does not affect forskolin potentiation. (*2'*) Affects forskolin potentiation only in Ionomycin-induced release.

Relating the model in the mossy fiber to the calyx of Held

Several parallels can be drawn between this model and the results of Mover knockdown in the calyx of Held (Körber et al., 2015). For example, similarly to the mossy fibers, the calyx of Held has its release properties influenced by cAMP (Sakaba and Neher, 2001b; Yao and Sakaba, 2010). cAMP enhancement of transmitter release has also been shown to be linked to Epac (Kaneko and Takahashi, 2004). Interestingly, G-protein-induced inhibition of Ca^{2+} entry in the calyx by AMPA receptor activation serves as a negative control for the model presented here (Takago et al., 2005): since the inhibition of calcium currents depends on $G_{\beta\gamma}$, and not on $G_{\alpha i}$, the process is independent of AC and would not be influenced by Mover. Indeed, calcium currents were unchanged in the Mover knockdown (Körber et al., 2015). However, in Körber et al. (2015), the authors argue that the observed effect of the knockdown is not due to CaM regulation of release. Nevertheless, that assumption was based on a pull-down assay of CaM and Munc13 in the presence of Mover, neglecting a possible indirect effect of Mover on the release machinery. This will be discussed below, in section 4.1.3 ‘Does Mover affect the release machinery?’.

Perspectives

This proposed model allows us to do further experiments to test it, as well as deepen our understanding of synaptic function. A first step would be verifying whether Mover inhibits the binding of CaM and AC1, AC8, or even AC3, via a pull-down assay.

Knockouts of RIM1α or Rab3A show an impaired LTP, while forskolin-induced potentiation is intact (Castillo et al., 1997, 2002). Similarly, it would be valid to test if the knockout of Mover would have an effect on electrically evoked LTP. Contrarily to RIM1α and Rab3A though, the prediction is that Mover’s presence would inhibit LTP, because of its inhibition of the AC/cAMP pathway. Therefore, the KO would either have a stronger LTP, which would be easily testable, or would have a lower threshold for LTP induction. The latter would require an LTP induction protocol that does not reliably induce LTP in the WT and test if the induction is more effective in KO animals.

To further verify the inhibition of Mover in the signaling cascade following the activation of Kainate receptors, a more specific Kainate receptor antagonist should be used. Electrophysiological experiments should be performed in the absence of the blockade and

have the antagonist wash in for an assessment of the kainate receptor block in both basal release and STP. Application of low concentrations of kainate (20-50 nM) could also give us a good insight on whether Mover is indeed blocking the downstream pathway. One should still keep in mind that presynaptic NMDA receptors might still influence the activation of AC and, therefore, be subject to Mover inhibition. However, the presence of these receptors in the mossy fiber terminal has only recently been discovered and the implications of NMDA manipulation there could be difficult to interpret (Lituma et al., 2016). Nevertheless, it could be an interesting and novel direction to be pursued.

4.1.2 Does Mover affect basal release probability?

In a knockdown study in the calyx of Held, Mover disruption was associated with an increased release probability due to a strengthened calcium sensitivity of release (Körber et al., 2015). In this study, the absence of change in the input-output curve, as well as a similar increase in response to different Ca^{2+} concentrations, argue towards an absence of change in basal release probability in the mossy fiber terminals of Mover knockout mice.

Curiously, here we observe an increase in paired-pulse ratio and in other facilitation responses, which is often correlated with a decrease in P_{vr}. The paired-pulse ratio is commonly used to assess changes in release probability (Manabe et al., 1993; and see Ben-Simon et al., 2015 for an example in the mossy fibers), in a way that a higher P_{vr} produces a smaller facilitation, or even depression. This can be explained by the model of depletion of the RRP (referred to in section 1.3.1.2 ‘Synaptic Depression’, see also Fioravante & Regehr, 2011). In this model, a greater P_{vr} would lead to a subsequent smaller number of available vesicles after an initial stimulus, leading to a smaller paired-pulse ratio, i.e. a smaller facilitation or a greater depression (see also Figure I.4). However, this correlation is not always true. An increase in facilitation can be brought about without a change in the initial P_{vr} (e.g. Jackman et al., 2016). Particularly, the synapse of mossy fiber to CA3 pyramidal cell has a remarkably low P_{vr} and an exceptionally large number of releasable vesicles, which renders it difficult to deplete the RRP (Hallermann et al., 2003). Furthermore, calcium buffer saturation also play a role in regulating facilitation in the mossy fiber terminals (Blatow et al., 2003; Vyleta and Jonas, 2014). Consequently, in this synapse, there is a rather weak dependence of the paired-pulse ratio on P_{vr} (Mori-Kawakami et al., 2003).

One simple way of increasing P_{vr} is by increasing the Ca^{2+} to Mg^{2+} ratio of the extracellular solution. Taking only the depletion model into consideration, an increase in calcium influx would lead to a decrease in paired-pulse ratio. However, this is not necessarily the case in this synapse. In Mori-Kawakami et al. (2003), increasing extracellular Ca^{2+} concentration only led to a slight decrease in paired-pulse ratio: a 12-fold increase in calcium concentration only changed the paired-pulse ratio by 40%. Stronger evidence comes from Blatow et al. 2003 where an increase in Ca^{2+} resulted in a surprising increase, instead of a decrease, in facilitation in the mossy fiber to CA3 pyramidal cell synapse. The results here corroborate this loose relationship between Ca^{2+} and the paired-pulse ratio: raising extracellular Ca^{2+} concentration from 1.25 to 2.5, and finally to 3.5 mM led to a change in paired-pulse ratio from approximately 2.2 to 2.4 to 1.2-fold (visible as the second data point in the train of stimuli in Figure $8A_2$-C_2). Nevertheless, in all three studies (this thesis; Blatow et al., 2003; Mori-Kawakami et al., 2003), an increase in extracellular Ca^{2+} concentration resulted in increased postsynaptic responses, expected from the calcium-driven increase in P_{vr}. Therefore, it is likely that, in the absence of Mover in mossy fiber terminals, the facilitation is changed without a change in initial P_{vr} (but further experiments are proposed in the subsection ‘Perspectives’, page 88).

No change in P_{vr} in mossy fibers versus increase in P_{vr} in the calyx of Held

Evidence points to the idea that the knockout of Mover in the mossy fibers increases the release of vesicles in an activity-dependent manner, with no change in basal (or initial) release probability. Why would there be no change in the initial P_{vr} in KO mossy fibers when there was an increase in P_{vr} in Mover knockdown in the calyx of Held (Körber et al., 2015)? There are several possible answers to that, though none are definite.

I have previously proposed that Mover plays a role in synaptic transmission by inhibiting the stimulation of AC by CaM. But AC is only one of many interaction partners of CaM in the presynaptic terminal. For example calcium channels (Catterall et al., 1999), Munc13 (Junge et al., 2004), and Calmodulin-dependent Kinase II (Popoli, 1993; Pang et al., 2010) are some other CaM interaction partners involved in synaptic vesicle release (see Xia & Storm 2005 or Mochida 2011 for a review). Therefore, if Mover is inhibiting the binding of CaM to other proteins in the calyx, this could explain the difference in initial release probability observed in the KO. However, I would like to dismiss some of these possibilities.

Firstly, Mover knockdown affected the calcium sensitivity of release, and not calcium influx (Körber et al., 2015). Therefore, calcium channels are unlikely to be affected. Secondly, the presence of Mover did not inhibit CaM-Munc13-1 binding in a pull-down assay with purified proteins (Körber et al., 2015). It is still possible that, *in vivo* or *ex vivo*, in the presence of other proteins in the presynaptic environment, this binding would be affected, but that is more difficult to be assessed. Lastly, inhibition of Calmodulin-dependent Kinase II in the calyx of Held affects the replenishment of the RRP but does not affect basal P_{vr} (Sakaba and Neher, 2001a; Sun et al., 2006). Curiously, knockdown of Calmodulin in cultured cortical neurons strongly decreases P_{vr} in an effect dependent on this kinase (Pang et al., 2010). However, since this does not seem to be the case for the calyx of Held, there is no evidence for this kinase to be the difference between the two reports on Mover disruption.

It is still possible that the difference in basal release probability is not brought about by CaM, but by a different Mover binding partner. Another known binding partner of Mover is a protein of the cytomatrix of the active zone: Bassoon (Kremer et al., 2007). Bassoon disruption has been associated with a reduction in RRP size and increase in P_{vr} in a synapse in the auditory pathway called the endbulb of Held (Mendoza Schulz et al., 2014). It is tempting to associate the binding of Bassoon and Mover with the changes observed in P_{vr}. However, it is possible that the change in P_{vr} in Mendoza Schulz et al. (2014) reflects a homeostatic adaptation to partial sensory deprivation. The reported reduction in P_{vr} can even be due to an observed reduction in Mover levels, which was also observed in the Bassoon disruption. Another major phenotype of Bassoon disruption is the slowing-down of vesicle replenishment (Hallermann et al., 2010; Mendoza Schulz et al., 2014). This effect was not significantly changed in the Mover knockdown and this, therefore, argues against Mover participation in this process.

Can the proposed model explain the different reports on P_{vr}?

An interesting hypothesis that could resolve the apparent paradox of Mover action in basal release probability in the hippocampal mossy fiber terminal (this thesis) and in the calyx of Held (Körber et al., 2015) lies in the model of Mover action in the cAMP pathway proposed here (Figure D.1).

Although AC1 is probably not present in the calyx of Held, it is known that stimulation of AC activity by forskolin also potentiates responses in this synapse (Xia et al., 1991; Sakaba

and Neher, 2001b; Yao and Sakaba, 2010). It is possible that some other isoform of CaM-sensitive AC is present in this brainstem synapse and mediates such a response: AC3 or AC8 (Wang and Storm, 2003; Halls and Cooper, 2011). Indeed, AC8 is strongly expressed in the brainstem, making it a good candidate (Muglia et al., 1999).

So far, these arguments only indicate that it is possible that the cAMP pathway could act in a CaM-dependent way in both the mossy fibers and the calyx of Held. So what could be differentially regulating this pathway to account for the possible difference in basal P_{vr} in Mover disruption in mossy fibers versus calyx of Held? One interesting possibility is the idea that AC activity is constitutively inhibited in mossy fibers and not in the calyx. One hypothetical link can be made: ambient adenosine levels lead to a tonic activation of A_1 adenosine receptors (Moore et al., 2003; Gundlfinger et al., 2007), whereas in the calyx of Held these receptors are involved in regulation of synaptic transmission but are not continuously active (Kimura et al., 2003).

Even though it has been reported that the activation of A_1 receptors leads to inhibition of Ca^{2+} channels (Kimura et al., 2003; Gundlfinger et al., 2007), it is still possible that it also affects AC activity: A_1 receptors have indeed been described to negatively regulate the cAMP pathway in several systems (van Calker et al., 1978; Ebersolt et al., 1983). The proposed double action of A_1 in regulating both Ca^{2+} channels and AC can be easily understood taking into consideration that this receptor is a G-Protein-coupled Receptor and G-proteins can inhibit Ca^{2+} channels with their $G_{\beta\gamma}$ subunits (Herlitze et al., 1996; Ikeda, 1996; Cantí et al., 1999) and inhibit AC by the $G_{\alpha i}$ subunit (Taussig et al., 1993). Nevertheless, the participation of the Adenosine receptor in the cAMP pathway in mossy fiber terminals should still be tested (see 'Perspectives' below).

Lastly, it is possible that the difference between the result on basal release probability in this study and Körber et al. (2015) is of a different nature. It is possible that, here, homeostatic plasticity effects might have played a role in returning the release probability of the synapse to a physiological level. In the calyx study, the approach was a knockdown and is, therefore, less likely to be subject to homeostatic plasticity.

Perspectives

Even though there is no apparent change in the basal P_{vr} in the KO mossy fiber terminals, it would be advisable to further investigate this in additional experiments. Changes in

presynaptic transmission properties at this synapse can be verified by assessing whether the KO changes the rate of synaptic failures upon minimal stimulation (Malinow and Tsien, 1990; Stevens and Wang, 1994), or the coefficient of variation through quantal analysis (Korn and Faber, 1991). Multiple-probability fluctuation analysis could also be used, though it has the caveat of requiring that the P_{vr} be varied over a large range, which makes it more suitable for high P_{vr} synapses (Clements and Silver, 2000; Lawrence et al., 2004).

Since the effect of Mover was both age- and calcium-dependent, it could prove useful to explore the range of extracellular calcium concentration in which the KO also produces a differential response in young mice. In this thesis, this was only done in adult mice.

Furthermore, on the topic of calcium sensitivity, the facilitation in the KO was slightly (non-significantly) smaller than in WT in the recordings with 1.25 mM extracellular Ca^{2+} (Figure 8A). It would be interesting to explore whether, at lower Ca^{2+} concentrations, the KO would actually lead to a weaker facilitation than in WT. This would suggest that Mover not only has a calcium-dependent effect, but is also involved a biphasic shift in the calcium-dependency of facilitation. Reduction in the extracellular Ca^{2+} concentration can be detrimental to the electrophysiological recordings at this synapse because of the low signal-to-noise ratio at lower concentrations. Nevertheless, recordings are still feasible (e.g. Mori-Kawakami et al., 2003).

The results provided here suggest that Mover is affecting vesicle release in a dynamic and Ca^{2+}-dependent way. Consequently, the idea of Mover affecting release inevitably leads to the question if this protein is affecting the release machinery. This will be discussed below.

4.1.3 Does Mover affect the release machinery?

The evidence that Mover directly influences the release machinery is lacking: Mover KO has unaffected basal synaptic transmission and Mover's known binding partners Bassoon and CaM are not molecules necessary for membrane fusion. However, the changes here presented in facilitation of transmitter release in the mossy fibers, together with the results observed in the knockdown in the calyx of Held (Körber et al., 2015), give enough support to the idea that Mover is indirectly affecting the release machinery. The hypotheses involving the effects of Mover's binding partners in release have been already discussed (page 86). Here I will examine some possible outcomes of Mover's presence in the presynaptic terminal.

One initial hypothesis of Mover's mechanism of action was based on the fact that it associates itself with Bassoon and with the synaptic vesicles. Bassoon was proposed to be a vesicle tether but does not seem to bind directly to them (Hallermann et al., 2010; Hallermann and Silver, 2013). The hypothesis was that Mover, binding to Bassoon, would regulate the distance between vesicle and the Ca^{2+} channel. In this way, Mover could be regulating P_{vr}. However, increased responses to calcium uncaging in Mover knockdown calyces argue against it (Körber et al., 2015). Calcium uncaging is independent of calcium influx through channels and, therefore, is not sensitive to changes in coupling distance.

4.1.3.1 Mover, Synaptotagmin 7 and Facilitation

Syt7 has recently been reported to be required for facilitation in many synapses, including the hippocampal mossy fibers (Jackman et al., 2016). This study from the lab of Wade Regehr suggests that Syt7 is the Ca^{2+}-sensor for facilitation. This view has been disputed in a study that argues that Syt7 contributes to facilitation by mediating asynchronous neurotransmitter release (Luo and Südhof, 2017). Nevertheless, the dramatic reduction in facilitation in the Syt7 knockout leaves no doubt that it influences activity-dependent release in the mossy fibers.

Since Mover's presence inhibits facilitation in the mossy fibers, whereas Syt7 promotes it, it is tempting to speculate that Mover would directly or indirectly inhibit Syt7. Further data suggesting that Syt7 accelerates (and Mover slows down) synaptic recovery after depression further support this hypothesis (Liu et al., 2014; Körber et al., 2015).

On the other hand, some recent data argues against this interaction. We have recently tested if tagged Mover and Syt7 colocalize when overexpressed in neurons and this does not seem to be the case (Thomas Dresbach, personal communication). This suggests the lack of a direct interaction between Mover and Syt7. Furthermore, knockout of Syt7 did not affect the release probability or short-term plasticity in the calyx of Held (Luo and Südhof, 2017). Facilitation, at low calcium concentrations, was unaffected. Other proposed roles for Syt7 such as asynchronous release (Luo and Südhof, 2017) and synaptic vesicle replenishment (Liu et al., 2014) were unchanged in Mover disruption (Körber et al., 2015). This body of evidence suggests that the interference of Mover in syt7-mediated exocytosis is improbable.

Perspectives

Overexpression of tagged Mover and Syt7 did not reveal a strong colocalization of the two proteins (Thomas Dresbach, personal communication), which hints towards the two proteins not interacting directly. However, this doesn't completely rule out that they interact since, for example, the tags (used for fluorescent visualization of the proteins) could have interfered with the binding. A different approach, such as a pull-down of the two proteins, could give further evidence of this possible interaction.

Independent of a direct binding between Mover and Syt7, further studies involving the two proteins could give interesting insights into their role as well as a better understanding of synaptic transmission and facilitation of neurotransmitter release. The knockout of Mover described here shows an increased facilitation, whereas the knockout of Syt7 shows an almost complete absence of facilitation (Jackman et al., 2016). In case Syt7 is indeed the sensor for facilitation a double-knockout of Syt7 and Mover would still lead to a starkly reduced facilitation, indistinguishable from the knockout of only Syt7. On the other hand, if Syt7 is not absolutely required for facilitation but only contributes to it via acting as a Ca^{2+} sensor for asynchronous release (as suggested by Luo & Südhof 2017), it is possible that this hypothetical double knockout would present a degree of facilitation between that of Mover knockout and Syt7 knockout. Moreover, such a result would also indicate that the effect through which the Mover knockout is increasing facilitation is not by disinhibiting Syt7, but through other mechanisms.

4.1.3.2 Mover, Rab3 and Superpriming

One of the downstream targets of Mover inhibition in the model proposed here is the protein Rab3 (Figure D.1). There are some interesting parallels between Rab3 and Mover. Firstly, they both affect P_{vr} without affecting the RRP size (Schlüter et al., 2004, 2006; Körber et al., 2015). Secondly, they both affect P_{vr} by changing the Ca^{2+} sensitivity of release (Schlüter et al., 2006; Körber et al., 2015). Thirdly, they both bind to synaptic vesicles and to proteins of the cytomatrix of the active zone: RIM and Bassoon, respectively (Wang et al., 1997; Kremer et al., 2007; Ahmed et al., 2013). Fourthly, inhibition of neuronal activity change the levels of both Mover and the Rab3 interaction partner RIM (Kremer, 2008; Lazarevic et al., 2011). Lastly, they have both been suggested to be involved in a process called superpriming (Schlüter et al., 2006; Körber and Kuner, 2016). Importantly, in all of the mentioned changes, Rab3 and Mover had opposite effects.

Mover appears to be inhibiting superpriming whereas Rab3 is promoting it (more on superpriming in the Introduction, page 23).

Rab3 forms a tripartite complex with RIM and Munc13 (Dulubova et al., 2005). One proposed mechanism through which Rab3 participates in superpriming is exactly through this interaction with Munc13 (Taschenberger et al., 2016). Munc13 has a C1 domain that, if interfered with, abolishes all potentiation by diacylglycerol (or analogues) by moving up the P_{vr} to an already-potentiated state (Basu et al., 2007). Furthermore, the whole C1-C2B module in Munc13 inhibits release, whereas binding of Ca^{2+} and diacylglycerol can mitigate this inhibition (Michelassi et al., 2017). Therefore, Munc13 provides a good substrate for the enhanced fusogenic state called superpriming (also supported by Ishiyama et al., 2014).

Taking into consideration the opposite effects of Mover and Rab3 described above, it is possible to propose that Mover is indeed inhibiting superpriming. It is even feasible that Mover is inhibiting superpriming through the proposed mechanism in the cAMP pathway, where Rab3 also takes part (Figure D.1). In the absence of Mover, disinhibiting this pathway would have led to an increased initial P_{vr} (Körber et al., 2015), or an increase in P_r that is activity-dependent (e.g. Figure 5).

However, one must proceed with caution in connecting superpriming, Mover and the cAMP pathway since there is also some evidence against it. It is important to state that, to the best of my knowledge, a connection between the cAMP pathway and superpriming has never been established before and this is not backed by strong experimental evidence. Instead, the connection is described by a correlative set of data involving this thesis and the model proposed here, the work of Körber et al. (2015), the involvement of cAMP in regulating Ca^{2+} sensitivity of release (Sakaba and Neher, 2001b; Yao and Sakaba, 2010), the similarity between effects of forskolin and phorbol ester in the mossy fibers (Kamiya and Yamamoto, 1997), and the involvement of Rab3 in superpriming and in regulating release in the mossy fibers through PKA or Epac (Castillo et al., 1997, 2002; Lonart et al., 1998; Schlüter et al., 2006; Fernandes et al., 2015).

One piece of evidence that argues against including superpriming in the proposed model is the participation of CaM. Lee et al. (2013) suggests that CaM does not participate in mediating superpriming, whereas in the model proposed here the interaction of CaM and AC is the locus of inhibition by Mover. Furthermore, their study suggests that the

maturation of a synaptic vesicle into a superprimed state is quite slow (τ=3.6 s). This time constant is not corroborated by the increased facilitation observed here in tens of milliseconds. Nevertheless, not all is lost. Considering that the mossy fiber bouton and the calyx of Held are quite different and that superpriming could be accelerated, it is still possible that Mover, cAMP and superpriming interact.

Perspectives

Both phorbol ester (analogue of diacylglycerol) and post-tetanic potentiation have been shown to promote superpriming (Taschenberger et al., 2016). Therefore, a simple initial experiment to test if Mover inhibits superpriming would be to test if the KO occludes the potentiation by phorbol ester or by post-tetanic potentiation.

Another occlusion experiment that could give evidence of cAMP having a role in superpriming would be the application of phorbol ester in slices pre-potentiated by forskolin. The potentiation provided by phorbol ester in this case should be smaller in case the pathway is already active by the increased cAMP levels by forskolin. In case cAMP does not lead to an increase in the proportion of vesicles that are superprimed, both potentiations should summate.

An increase in cAMP levels in the calyx of Held has been linked to an increase in the Ca^{2+} sensitivity of release of the fast-releasing vesicles, with no apparent effect on the coupling distance (Yao and Sakaba, 2010), an effect conspicuously similar to that described as superpriming (Lee et al., 2013; Taschenberger et al., 2016). Yao & Sakaba (2010) even state that the effect of forskolin is similar to that of phorbol esters described in Lou et al. (2005). It would be, therefore, interesting to reevaluate the results from Sakaba & Neher (2001b) and Yao & Sakaba (2010) under the light of superpriming to assess the possibility of the cAMP pathway to be involved in this process.

4.1.3.3 Mover, the SNARE complex and Schizophrenia

Munc13 has long been known to participate in vesicle release but has recently been given particular importance in regulating proper SNARE complex assembly (Betz et al., 1998; Augustin et al., 1999; Lai et al., 2017). Proteins of the SNARE complex have also been reported to be altered in the brains of schizophrenic patients (e.g. Honer et al., 1997; Gabriel et al., 1997). Interestingly, a recent study shows increased SNARE complex

formation in postmortem brain samples of schizophrenic patients (Ramos-Miguel et al., 2015).

Mover levels have also been reported to be strongly increased in the cingulate cortex of schizophrenic patients (Clark et al., 2006; Mover referred to as Novel Protein RP11-46F15.3). This is the same area in which some of the alterations in SNARE complex have been described (Gabriel et al., 1997; Honer et al., 1997; Ramos-Miguel et al., 2015). It is possible to speculate that increased Mover levels and increased SNARE complex formation are related in some way. Additionally, Calcineurin, which dephosphorylates Mover (Mahdokht Kohansal-Nodehi, personal communication), has also been associated with Schizophrenia (Miyakawa et al., 2003).

At this point, it is difficult to predict if the increase in Mover levels are a consequence of the disease or part of its cause. Since Mover has a proposed activity-dependent expression (see section ‘Age-dependent effect and activity-dependent expression of Mover’ on page 78), one could predict that Mover would have increased presence in this area in case there was stronger activity there in schizophrenic brains. Mover presence could, therefore, lead to a decrease in activity, in an attempt to reestablish basal levels of activity (see also section 4.2.2 ‘Mover dynamically buffers synaptic strength’ below). However, this might not be the case as there is an apparent decrease in glutamate concentration (Gallinat et al., 2016) and hypoactivity in the cingulate cortex, though this differs depending on the task being executed and the specific region looked at (Adams and David, 2007). Hypoactivity in the cingulate cortex of schizophrenic patients would argue for a decrease, and not an increase, in Mover expression. Hence, this argues against Mover upregulation being a homeostatic plasticity response to increased activity in this brain region. On the other hand, it is not known whether the cingulate cortex of schizophrenic patients is hypo- or hyperactive during their everyday lives. Furthermore, nothing is known about the distribution of Mover in the neurons in this area. It is very well possible that Mover is present at inhibitory synapses and therefore, the expected regulation would be indeed an increase in Mover levels. These are deliberations on the hypothesis that Mover is increased as a consequence of schizophrenia.

On the other hand, if Mover is part of the cause of schizophrenia, it is possible to contemplate the idea that increased Mover presence is involved in the increased SNARE complex formation (Ramos-Miguel et al., 2015). However, at this point, this is all

speculative and experiments should be done in this direction to further understand the interactions between Mover, SNARE complexes and schizophrenia.

Perspectives

A simple way to start verifying the interaction of Mover with schizophrenia would be to make use of mouse models of schizophrenia in which the SNARE complex is implicated, such as the blind/drunk mouse model (Jeans et al., 2007; Oliver and Davies, 2009). In this model, a point mutation in SNAP25 increases the stability of the SNARE complex, similar to what was observed in postmortem samples from human brain (Ramos-Miguel et al., 2015). An initial approach to assess whether the increased Mover presence in the brain of schizophrenic patients (Clark et al., 2006) is a result of SNARE complex misbehavior would be to analyze if the expression of Mover is altered in these mice. It could also prove useful to test via immunohistochemistry if this increased expression also translates into increased Mover presence in the synaptic terminals.

However, if Mover is not the consequence but the cause for the observed changes in SNARE complex formation, it is likely that no changes in Mover's presence would be observed in models such as the blind/drunk mouse model. On the other hand, if Mover is to be implicated in the etiology of schizophrenia it would be interesting to verify if a Mouse model with Mover overexpression would exhibit schizophrenia-related endophenotypes.

If Mover is increasing SNARE complex formation or stabilizing those complexes, it would be possible to test that with biochemical assays. The formation of the complex could be tested with different Mover concentrations to test whether Mover indeed plays a role.

4.2 Hypotheses for Mover's Functional Implications

4.2.1 Mover dampens synaptic temporal-filtering

Mover is a vertebrate-specific protein (Kremer et al., 2007). As a vertebrate-specific protein, it is unlikely that Mover would be required for basic mechanisms, such as vesicle fusion, in which most proteins are conserved across phylogeny (Ackermann et al., 2015).

As expected, the results presented here suggest that Mover has a regulatory role in facilitation or in vesicle release. It is possible to draw an initial conclusion on how the

regulation by Mover plays a role in the bigger picture: the presence of Mover affects synaptic computation. As discussed in the introduction about Synaptic Plasticity (section 1.3), short-term plasticity can act as temporal filters. Facilitating synapses can act as high-pass filters, while depressing synapses act as low-pass filters. And Mover loosens the synaptic filter.

The presence of Mover in the strongly facilitating synapse, the hippocampal mossy fiber to CA3 pyramidal cell synapse, reduces facilitation (Figure 5). In a depressing synapse, the calyx of Held, Mover was associated with a weakening of the depression (Körber et al., 2015). In the Schaffer collaterals, a synapse with much less prominent STP than the two mentioned above, Mover presence did not interfere with synaptic plasticity (Figure 3). Therefore, Mover reduces plasticity in synapses with strong STP and, by doing so, diminishes their temporal filtering properties.

This hypothesis is, however, only based on results from three synapses, and should be approached in future studies.

Perspectives

To further verify whether Mover is indeed generally acting as a break in STP, the effect of the knockout needs to be verified in other synapses. The cerebellar mossy fiber to granule cell synapse, for example, is a synapse with similar structure to the hippocampal mossy fiber bouton but strongly depresses (Delvendahl et al., 2013). This could be a good candidate to measure if Mover knockout also increases depression, provided that Mover is present in this synapse. An increase in depression in this synapse would, however, be quite a feat, since this synapse has already a particularly high P_{vr} (Hallermann et al., 2010). To verify that the KO decreases facilitation in other facilitating synapses, recordings from the thalamocortical synapses between layer 6 cortical pyramidal cells and thalamic relay cells can be performed with stimulation in the internal capsule (e.g. Jackman et al., 2016).

To test if Mover is indeed having the suggested effect of temporal filtering, it would be interesting to test how the KO affects spiking of the postsynaptic neuron in response to different stimulation protocols.

4.2.2 Mover dynamically buffers synaptic strength

The idea that Mover might be involved in mechanisms such as temporal filtering and in diseases such as schizophrenia (see page 93) relates well with the notion that Mover regulates synaptic strength. I have shown that Mover reduces facilitation in a synapse where transmission relies heavily on this increase in efficiency (Vyleta et al., 2016). On the other hand, Mover reduces the amplitude of the first response to a train of stimuli, in a synapse where this initial response is vital for transmission of fast and reliable auditory information (Englitz et al., 2009; Körber et al., 2015). Taken together, these results suggest that Mover buffers synaptic strength.

Furthermore, when taking into consideration that Mover is downregulated in reduced neuronal activity (Kremer, 2008; Mendoza Schulz et al., 2014; Wetzel, 2015) and upregulated in increased neuronal activity (discussed on page 78; Moritz Arndt, personal communication), it is possible to propose that Mover is dynamically regulating synaptic strength.

The activity-dependent expression of Mover, in connection to its role in buffering synaptic strength, makes it a perfect candidate for being a tool in homeostatic plasticity. Homeostatic plasticity is a neuronal compensatory adjustment in response to overall network activity in order to keep neuronal firing rates in a physiological range (Lazarevic et al., 2013).

When neuronal activity is weakened by either pharmacological agents (Kremer, 2008) or defective neuronal transmission in the upstream synapse (Mendoza Schulz et al., 2014; Wetzel, 2015) Mover levels went down. Reduction of activity often leads to a compensatory increase in quantal size and in P_r (Lazarevic et al., 2013). A reduction in the presence of Mover can also lead to an increase in P_r (Körber et al., 2015). Consequently, it is possible to speculate that the reduction in Mover levels during homeostatic adaptation is partially responsible for the change in P_r. Mover could, therefore, contribute to the manifestation of homeostatic plasticity.

This proposed dynamic regulation of synaptic strength relates well to the topic of Mover upregulation in the brains of schizophrenic patients (discussed in section 4.1.3.3 ‘Mover, the SNARE complex and Schizophrenia’). If Mover does indeed play a role in dynamically buffering synaptic strength, it might also have implications in other neurological diseases

such as epilepsy. On a cellular level, Mover reducing synaptic strength might help prevent excitotoxicity and, therefore, have a neuroprotective effect.

Perspectives

Verifying the proposed roles of Mover in dynamically regulating synaptic strength would be an important step to further understand its role, but could also lead to important discoveries in the field of neuroprotection. Therefore, it would be interesting to use the Mover knockout mouse line to investigate whether Mover presence acts in preventing cell death due to excitotoxicity. This could be done by inducing the neurons to a high excitability state and verifying the amount of apoptotic cells, for example via the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, in WT versus KO.

It would be also important to examine whether, in KO mice, epileptic-like activity is more easily triggered. The idea behind this is that epileptic activity can be the result of too much excitation in the brain and, therefore, the increased synaptic strength in the absence of Mover could make it easier to initiate seizures. However, epileptic activity can also depend on the balance between excitation and inhibition. If the lack of Mover is increasing release from inhibitory synapses, it is possible that no effect will be observed in this experiment. Accordingly, the effect of mover knockout should also be studied in inhibitory terminals.

Furthermore, additional studies on the role of Mover in homeostatic plasticity should be carried out. Additional experiments, mainly in situations of increased neuronal activity, should be carried out to confirm the upregulation of Mover in this situation. This increased excitability of cells can be achieved in different ways, such as increased K^+ concentration and blockade of GABA receptors. It is noteworthy to state that neuronal cultures have often very decreased neuronal activity due to the culture medium (Bardy et al., 2015). Hence, homeostatic plasticity changes studied in cultured cells could possibly better reflect physiological processes if the neurons were raised in a medium more supportive of action potential generation and synaptic transmission. Additionally, it would be interesting to test not only Mover expression but also if its intensity in synapses is also enhanced.

Lastly, in case Mover is indeed contributing to the changes observed in homeostatic plasticity, it should be assessed whether the changes normally observed in P_r due to inhibition of activity are affected in the absence of Mover. If Mover plays a role in

increasing the P_r in situations of extended neuronal silence the increase in P_r should be attenuated in the KO.

4.2.3 Mover could affect memory formation

Both the hippocampal mossy fibers and short-term plasticity have been implicated in memory and cognition (e.g. Gilbert & Kesner 2006; Kesner 2007; Mongillo et al., 2008; Nakashiba et al., 2008; Farovik et al., 2010; Liu et al., 2013). Therefore, it is possible that Mover influences cognition.

It is believed that synaptic plasticity is the building block from which memories are formed (e.g. Bliss & Collingridge, 1993; Poo et al., 2016). Recent evidence suggests that STP is the substrate for a short-term memory referred to as working memory (Mongillo et al., 2008). Interfering with synaptic transmission affects working memory (e.g. Franowicz et al., 2002). Remarkably, activation of PKA, which participates in the model described in this thesis (Figure D.1), leads to impairment of working memory (Taylor et al., 1999). However, experiments in mice that lack Rab3A, which acts downstream of PKA, failed to show an impairment in working memory (Hensbroek et al., 2003). Then again, the knockout of Rab3A shows defective LTP, but no effect on STP, which is the locus often described for working memory (Castillo et al., 1997; Mongillo et al., 2008).

Working memory has been more widely associated with the prefrontal cortex (Goldman-Rakic, 1995; Mongillo et al., 2008), but experiments also show a participation of the hippocampus, mostly in spatial memory (e.g. Kesner, 2007; Vago & Kesner, 2008; Jadhav et al., 2012). Therefore, it is possible that the knockout of Mover affects working memory by affecting STP in the hippocampus.

Furthermore, the connection between the dentate gyrus and the CA3 participates in a process called pattern separation (Kesner & Rolls, 2015; Knierim & Neunuebel, 2016; also introduced in section 1.4 ‘The Hippocampus’). The change in synaptic transmission observed in the absence of Mover can be supposed to affect pattern separation. Activation of the trisynaptic pathway (DG, CA3, CA1) is also important for pattern completion and could, therefore, be altered in the KO (Nakashiba et al., 2008; Guzman et al., 2016).

Perspectives

Some of the first experiments connecting synaptic plasticity to some kind of learning were done in the sea slug *Aplysia* (reviewed in Kandel, 2001). In this mollusk, touching its siphon leads to a reflex that withdraws its gill and siphon. A presynaptic enhancement of neurotransmitter release in its sensory to motor neuron synapse leads to a stronger withdrawal. Likewise, habituation, and therefore reduction of the behavioral response, is a result of a decrease in transmitter exocytosis. However, the connection between synaptic response and behavioral response is not always so isomorphic (Takeuchi et al., 2013). Hence, an increase in facilitation could theoretically lead to an improvement in cognitive performance, but this is not necessarily the case. This could be verified using the Mover knockout mice for behavioral experiments.

Behavioral experiments with Mover knockout mice could aim at assessing if spatial working memory, pattern separation and pattern completion are affected (as discussed above). Nevertheless, it is not known whether the absence of Mover affects long-term plasticity or even if it affects other synapses involved in cognition. Therefore, behavioral experiments that encompass other forms of memory should also be performed. And further electrophysiological experiments could unravel the involvement of Mover in LTP and in synaptic transmission in other synapses.

Mover could even provide a tool for better understanding memory. The lack of effect of the KO in the Schaffer collaterals provides us with a tool to better dissect mossy fiber-specific behavior and if future experiments show that there is no effect on LTP, the KO would also allow for studying of STP-specific behavior. Remarkably, the knockout of Mover might be one of the only easily accessible genetic tools to further increase facilitation in the mossy fibers without affecting other factors. This makes it a prime tool for understanding the purpose of such a prominent feature as the facilitation of the mossy fiber synapses. Nevertheless, a conditional knockout of Mover could prove useful in order to prevent off-site effects.

In conclusion, further studies on Mover could lead to not only a better understanding of Mover function and synaptic transmission but could also deepen our knowledge of memory formation, homeostatic plasticity and even neurological or psychiatric diseases such as schizophrenia.

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7 List of Abbreviations

AC	Adenylyl cyclase	mEPSC	Miniature excitatory postsynaptic current
ACSF	Artificial cerebrospinal fluid	mGluR	Metabotropic glutamate receptor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
CA	Cornu Ammonis	NMDA	N-methyl-D-aspartate
CaM	Calmodulin	PCR	Polymerase chain reaction
cAMP	Cyclic adenosine monophosphate	PKA	Protein kinase A
DCG-IV	2S,20R,30R-2-[20,30-dicarboxycyclo-propyl]glycine)	P_r	Synaptic release probability
DG	Dentate gyrus	PSC	Postsynaptic current
Epac	Exchange protein directly activated by cAMP	P_{vr}	Vesicular release probability
EPSC	Excitatory postsynaptic current	RIM	Rab-interacting molecules
fEPSP	Field excitatory postsynaptic potential	RRP	Readily releasable pool
GABA	Gamma-aminobutyric acid	STP	Short-term plasticity
ISI	Inter-stimulus interval	TTX	Tetrodotoxin
KO	Mover knockout mice	VGCC	Voltage-gated calcium channel
LTP	Long-term potentiation	WT	Wildtype mice

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9 DECLARATION

Herewith I declare, that I prepared the doctoral thesis "The presynaptic protein Mover buffers synaptic plasticity at the hippocampal mossy fiber synapse" on my own and with no other sources and aids than quoted.

Göttingen, 29th of September 2017.

_____

Julio Santos Viotti

10 CURRICULUM VITAE

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