Long-term synaptic plasticity is occluded at Schaffer collateral synapses from Synaptophysin-family knockout mice because of increased probability of vesicle release

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List of abbreviations

µm	Micrometer
µM	Micromolar
ACSF	Artificial cerebrospinal fluid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP	Action Potential
APV	(2R)-amino-5-phosphonovaleric acid
Arc	Activity-regulated cytoskeleton-associated protein
AZ	Active Zone
BDNF	Brain-derived Neurotrophic Factor
CA	*Cornus ammonis*
CaM	Calmodulin
CaMKII	Ca^{2+} /calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CNS	Central Nervous System
CPEB	Cytoplasmic polyadenylation element binding
CREB	cAMP response element-binding protein
E.g.	Exempli gratia
E-LTP	Early Long-Term Potentiation
EPSC	Excitatory Postsynaptic Current
EPSP	Excitatory Postsynaptic Potential
fEPSP	field Excitatory Postsynaptic Potential
GABA	γ-Aminobutyric acid
GPCR	G protein-coupled receptor
Hz	Hertz
KO	Knockout
Kyn	Kynurenic acid
L-LTP	Late Long-Term Potentiation
LTD	Long-Term Depression
LTP	Long-Term Potentiation
M	Molar
mEPSC	miniature Excitatory Postsynaptic Current
mM	Millimolar
MNTB	Medial nucleus of trapezoid body

mOsm	Milliosmole
ms	Millisecond
nA	Nanoampere
nm	Nanometer
NMDAR	*N*-methyl-D-aspartate receptor
PK(A/C/M)	Protein Kinase (A/C/M)
PP	Phosphatase
PSD	Postsynaptic density
PTP	Post-Tetanic Potentiation
p_v	Probability of release per vesicle
QKO	Quadruple knockout
RRP	Readily Releasable Pool
RT	Room Temperature
SNAP	Soluble N-ethylmaleimide-sensitive factor protein
SNARE	Soluble N-ethylmaleimide-sensitive factor activating protein receptor
STD	Short-Term Depression
STP	Short-Term Plasticity
Syp	Synaptophysin
SYT	Synaptotagmin
TARP	Transmembrane AMPA receptor regulatory protein
VAMP2	Vesicle Associated Membrane Protein 2
VCN	Ventral cochlear nucleus
VGCC	Voltage-Gated Ca^{2+} Channel
WT	Wildtype

Abstract/Resumen

Synapses are the connections between neurons that allow for the exchange of information. These connections involve various molecules, such as the Synaptophysin family, a group of four proteins embedded into the membrane of vesicles stored in the presynaptic terminal. Information is transmitted from a presynaptic terminal to a postsynaptic side via neurotransmitters, which are secreted by vesicle exocytosis. Our lab has previously shown that the deletion of the entire Synaptophysin family in mice (known as the quadruple knockout or QKO) results in stronger synapses due to an enhancement in the probability of release (p_v).

Several factors influence the size of the synaptic response, with some located in the presynaptic side and others in the postsynaptic. On the presynaptic terminal, we have the readily releasable pool size, which determines the number of vesicles available for neurotransmitter release, and p_v, which is the probability with which each readily releasable vesicle undergoes exocytosis following an individual action potential.

Previous research has shown that the deletion of just two members of the Synaptophysin family can lead to abnormalities in synaptic plasticity, which is the capacity of synapses to undergo functional changes depending on the frequency of use. These changes can enhance or reduce the synaptic response and can be categorized into two groups based on their duration: Short- and Long-Term Plasticity. Short-Term Plasticity may be related to computation tasks such as information filtering or spatial perception, while Long-Term Plasticity is thought to underlie learning and memory. Both are essential for brain function, so given the background results showing the effects of Synaptophysin family member deletion on plasticity, it is necessary to investigate how this is altered at QKO synapses.

Regarding Long-Term Plasticity, we attempted to potentiate synaptic responses in ex vivo slices from QKO and wildtype mice hippocampus. Although both got potentiated, after a while, synaptic responses returned to their initial levels in QKO. Lowering p_v by reducing calcium concentration rescued the potentiation in QKO. We even observed how enhancing p_v by increasing calcium at wildtype synapses caused the same occlusion in potentiation as seen in QKO. This clarifies that the elevated baseline p_v at QKO synapses is the cause of the potentiation occlusion. The underlying mechanisms are intact, and synaptophysin proteins do not play an essential role.

The generality of this QKO phenomenon need to be checked, so Short-Term Plasticity was also tested. Short-Term Potentiation events raise the p_v, but since QKO already have an increased p_v, it could not increase as much as in wildtype mice. Nonetheless, this phenotype was rescued again by reducing the calcium concentration in the preparations.

Altogether, these results identify p_v as the target factor responsible for the

modifications in the synaptic response expressed in Short- and Long-Term Plasticity. Furthermore, we can say that the limit enhancement in p_v is shared between QKO and Long-Term Plasticity.

Las sinapsis son conexiones entre neuronas que permiten la transmisión de información. Estas conexiones involucran varias moléculas, como las Sinaptofisinas, una familia de cuatro proteínas incrustadas en la membrana de vesículas almacenadas en el terminal presináptico. La información es transmitida desde el terminal presináptico al postsináptico a través de neurotransmisores, que son secretados durante la exocitosis vesicular. Investigaciones previas de nuestro laboratorio mostraron como la deleción de la familia de Sinaptofisinas en ratones (conocido como cuádruple knockout o QKO) resulta en transmisiones sinápticas más fuertes debido a un incremento en la probabilidad de liberación vesicular (p_v).

Varios factores influyen en el tamaño de la respuesta sináptica, algunos ubicados en el terminal presináptico y otros en el postsináptico. En el presináptico tenemos el tamaño del contingente de vesículas listas para ser liberadas, que determina la cantidad de vesículas disponibles para la liberación de neurotransmisores, y la p_v, la probabilidad de cada una de esas vesículas del contingente de ser exocitada tras un potencial de acción.

Investigaciones previas han mostrado como la deleción de solo dos miembros de la familia de las Sinaptofisinas puede llevar a anomalías en la plasticidad sináptica, la capacidad de las sinapsis que les permite sufrir cambios funcionales acorde a su frecuencia de uso. Estos cambios pueden aumentar o disminuir la respuesta sináptica y pueden ser divididas en dos grupos en función de su duración: plasticidad a corto y largo plazo. La plasticidad a corto plazo podría estar involucrada en tareas de computación como filtrar información o percepción espacial, mientras la plasticidad a largo plazo compondría la memoria y el aprendizaje. Ambas son esenciales para el funcionamiento del cerebro, y debido a que investigaciones previas muestran los efectos de eliminar miembros de la familia de Sinaptofisinas en plasticidad, es necesario investigar cómo esto afecta a las sinapsis de los QKO.

Respecto a la plasticidad a largo plazo, intentamos potenciar las respuestas sinápticas en rodajas de hipocampo de ratones QKO y control. Ambas fueron potenciadas, pero tras un tiempo las respuestas volvieron a los niveles iniciales en QKO. La disminución de la p_v gracias a reducir la concentración de calcio recuperó la potenciación en los QKO. Incluso vimos como incrementar la p_v gracias a aumentar el calcio en las sinapsis de ratones control provocaba la misma oclusión en la potenciación. Esto aclara que la elevada p_v de las sinapsis en QKO es la causante de la oclusión en la potenciación. El mecanismo subyacente se mantiene intacto y la familia de Sinaptofisinas no juega un papel esencial.

La generalidad de este fenómeno en los QKO necesitaba ser comprobada, por lo que la plasticidad a corto plazo fue estudiada. La potenciación a corto plazo eleva la p_v, pero

como los QKO ya la tienen incrementada, esta no podía elevarse tanto como en los ratones control. Sin embargo, el fenotipo fue rescatado de nuevo gracias a reducir la concentración de calcio en las preparaciones.

En conjunto, estos resultados señalan a la p_v como el factor responsable de las modificaciones en la respuesta sináptica consecuentes de la plasticidad a corto y largo plazo. Además, podemos decir que el límite en el crecimiento de la p_v es compartido entre los QKO y la plasticidad a largo plazo.

Introduction & Objectives

Communication is a fundamental action that supports life. It does not matter whether it is between people, a signal from a computer to another, or, the topic that concern us: neurons. The world around us is perceived by specialized nerve cells; light,sounds, odors… all that information is conveyed by cells into neural signals, which are transmitted to the brain thanks to other neurons. The brain is a complex formed by glial cells and neurons connected to each other, exchanging information continuously. Interactions among neurons process information from both environment and ourselves, enabling thinking. This communication between neurons is carried out via synapses, and their capacity to change is key for the brain function.

Santiago Ramón y Cajal's *neuron doctrine* was the first to propound that the brain is formed by individual interconnected cells, in this case, neurons, instead of continuous tissue, as had been thought for decades. For this work, Ramón y Cajal is widely considered to be the first Neuroscientist, and was awarded the Nobel Prize in Medicine and Physiology in 1906.

Ramón y Cajal was working before the invention of the electron microscope, and was never able to see the space between neurons, now termed the *synaptic cleft*. However, he was able to deduce that neurons communicate with each other via thin cellular processes now termed axons and dendrites.

Some years later, Sir Charles Scott Sherrington and his teacher Sir Michael Foster showed how the junctions between neurons could transmit electrical signals, and coined the term "synapse", in 1897. These studies, together with others, would earn the Nobel Prize in Medicine and Physiology for Sherrington in 1932.

In synapses, one neuron "sends" information and another receives it. The subcellular compartment that sends the information is called the presynaptic terminal and the compartment that receives the information are the postsynaptic elements.

There are two kinds of synaptic communication according to how information is transmitted: electrical synapses, where the cell membranes (or plasmalemmas) are physically connected through channels formed by proteins called connexons. These channels are termed gap junctions and are permeable to ions, allowing voltage changes in the presynaptic neuron to directly drive voltage changes in the postsynaptic cell (Bennett & Zukin, 2004).

The great majority of synapses, however, are chemical synapses. In this case, plasmalemmas are not directly in contact,even though the presynaptic terminal and postsynaptic element are close to each other. Instead, chemical molecules, known as

neurotransmitters, are released from the presynaptic terminal into the *synaptic cleft*, a space of 20-30 nanometers (nm) between the two cells. There, the neurotransmitter will bind to receptors on the postsynaptic element, causing a variety of actions, from voltage changes to protein cascade events.

There are many factors that influence the strength of chemical synaptic transmission - such as the amount of neurotransmitter released and the number of receptors on the postsynaptic side - and the speed. Most of the factors can be modified and changes lead to differences in the postsynaptic response. This capacity of synaptic transmission for change is called plasticity.

Thanks to strengthening or weakening of synaptic responses we have many major brain functions, like language, feelings, or memory. Plasticity is the basis for remembering and forgetting things. The way our brains implement memories is by modifying synapses. Despite being a main field in Neuroscience, there are still many questions about plasticity. For example, which are all the factors involved or how it is modulate.

In this thesis, I will study one of the elements implied in plasticity: the probability of release of the presynaptic vesicles that are docked and primed at release sites on the inside of the plasmalemma of presynaptic terminals (p_v). Understanding its role and how it relates with other factors is crucial for gaining insight into the overall phenomenon of plasticity.

1. Cell Biology of the chemical synapse

Plasticity modifications can occur at a variety of stages of the synaptic transmission, from the initiation of neurotransmitter release in the presynaptic terminal to the effects provoked in postsynaptic side. While my research focuses on the probability of release, (a factor determined by elements within the presynaptic terminal) comprehending the entire process of synaptic transmission is essential to study a specific component of it.

1.1 Presynaptic terminal

Synaptic transmission begins with the depolarization of the presynaptic terminal. This depolarization is triggered by the arrival of an action potential, an electrical wave that travels along the cell membrane of the presynaptic neuronal axon. The action potential raises the membrane potential from its resting level of -70/60 millivolts (mV) to above

+40 mV. As a result, the presynaptic cell undergoes depolarization, and voltage gated Ca^{2+} channels (VGCC) within the plasmalemma of the terminal open. This opening allows Ca^{2+} ions flowing into the terminal from the outside due to a concentration difference with the extracellular medium. The Ca^{2+} levels outside the cell are more than 10^4 times higher than inside (Simons, 1988). An increase in intracellular Ca^{2+} concentration initiates a mechanism for the release of neurotransmitters into the synaptic cleft.

Neurotransmitters are chemical messengers stored inside vesicles in the presynaptic terminal. Their release occurs at the active zone (AZ) of the presynaptic terminal membrane, place where some of the whole vesicles are docked for going exocytosis. However, it is unknown if all the docked vesicles are in the “primed state” necessary for exocytosis. Primed vesicles are competent for immediate fusion with the plasmalemma and await only Ca^{2+} entry to initiate exocytosis process. Proteins such as Munc13 and Complexin play important roles in priming, and their activation potentiates the neurotransmitter release by increasing the number of vesicles ready for exocytosis (Lai et al., 2017; Lou et al., 2008).

Synaptobrevin 2 (also known as VAMP2), a protein integrated within the vesicle membranes, forms a helix with two other proteins located in the plasmalemma AZ: Syntaxin and SNAP25. Together, these three, linked by their α-helix domains, constitute the core of the SNARE complex (Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptor). This group of proteins plays a crucial role in mediating vesicle exocytosis (Rizo, 2018).

This exocytosis process can occur spontaneously with a low frequency. However, the cooperative model proposed by Dodge & Rahamimoff (1967) suggests that synaptic strength depends on Ca^{2+} levels in a non-linear fashion, explained by the requirement of 4 Ca^{2+} ions for each release event. Since SNARE complex cannot bind Ca^{2+}, there must be a Ca^{2+} sensor that interacts with it. The most consensual candidate for this role is the protein Synaptotagmin (SYT), proposed by Thomas Südhof and colleagues. Südhof, along with James Rothman and Randy Schekman, were awarded the Nobel Prize in 2013 for their discoveries related to vesicle trafficking (Wickner, 2013).

The SYT family of proteins is characterized by their two C2-domains (C2A and C2B) and their interaction with the SNARE complex. The family contains 17 members. Of these, SYT1 is the most studied, and is widely accepted as playing a key role in coupling Ca^{2+} to exocytosis. SYT1 can bind up to 5 Ca^{2+} ions (three for C2A-domain

and two for C2B), which would initiate the exocytosis process (Fernández-Chacón et al., 2002). SNARE complex and SYT1 are close together in primed vesicles, and Ca^{2+} binding to C2-domains induces a conformational change in the SNARE complex (Ullah et al., 2021; Wolfes & Dean, 2020).

So, when an action potential arrives to the presynaptic cell, allowing Ca^{2+} entry, it binds to SYT1, causing a conformational change in SNARE complexes. This will close together the vesicle membrane and the plasmalemma, which will fuse due to proximity. The process will finish with the exocytosis of the vesicle releasing the neurotransmitter stored inside into the synaptic cleft (Südhof, 2004).

1.2 Postsynaptic compartment

Synapses can be classified based on how the postsynaptic cell responds to neurotransmitter release. Excitatory synapses result in an elevation of the postsynaptic membrane potential, bringing it closer to the action potential threshold. In another way, inhibitory synapses (e.g., GABA) prevent the enhancement of the membrane potential, hindering depolarization. This hindrance involves different events, such as the entry of Cl^-, the exit of K^+, or protein cascades, among other processes. If a synapse is excitatory or inhibitory depends on the type of neurotransmitter that is released and the type of receptors it binds to.

There are over 100 distinct types of neurotransmitters that can be released from the presynaptic terminal, depending on the examined synapses (Cuevas, 2019). Additionally, there are several kinds of receptors to which each can bind. Receptor proteins can be classified into two broad families that differ in their mechanisms of transducing neurotransmitter binding into postsynaptic responses: ionotropic and metabotropic receptors. Ionotropic receptors are channels that allow the flow of ions once activated by the neurotransmitter. In contrast, metabotropic receptors lack channels; a part of their structure is an intracellular domain that can activate a variety of signaling cascades after neurotransmitter binding, such as G-protein coupled receptors (GPCRs) (Lefkowitz, 2007).

The most important neurotransmitter mediator of excitatory signals in the central nervous system (CNS) is glutamate, which is used by more than half of the synapses in mammalian brains. After being released by exocytosis into the synaptic cleft from the vesicles, glutamate can bind on the postsynaptic side to both ionotropic and metabotropic receptors (Zhou & Danbolt, 2014).

In this thesis, I will study responses caused by ionotropic receptors that, once activated by glutamate, open and allow Na^+ and K^+ to flow in and out respectively. The exchange of ions after channel opening induces changes in the voltage across the cell membrane, which is the main synaptic response. If the membrane voltage reaches the threshold for firing action potentials, the postsynaptic neuron will initiate another synaptic transmission to their own postsynaptic target neurons, being this time the presynaptic side.

Three kinds of glutamate ionotropic receptors are named according to which artificial agonist is most specific. The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) are primarily responsible for postsynaptic depolarization. They are tetramers, each composed of two dimers, which, in turn, are composed of two of four types of subunits (Kamalova & Nakagawa, 2021). N-methyl-D-aspartate receptors (NMDARs) are normally blocked by Mg^{2+} ions, so their contribution to postsynaptic responses tends to be minor when the postsynaptic neuron is resting. However, if the Mg^{2+} blockade is removed due to postsynaptic depolarization, NMDARs play an important role in plasticity (Pérez-Otaño et al., 2016). Kainate receptors, or kainic acid receptors, are not extensively defined, and a significant number of them are found on the presynaptic side (Valbuena & Lerma, 2021). Therefore, I will leave aside their role in the synaptic response.

1.3 Synaptic response measurement

Each time the postsynaptic receptors are open, ions flow in and outside the cell causing changes in the voltage of the postsynaptic neuron. By connecting a sharp electrode in contact with the interior of the neuron to an amplifier these changes can be measured, allowing the electrophysiological recordings.

If the amplifier is in “voltage clamp mode”, it holds cell membrane constant by continuous injection of current. The holding will balance the current that comes into the cell through its receptor channels. The amplifier will output a voltage signal that is directly proportional to how much current it is injecting. This measurement is called a Post Synaptic Current, or Excitatory Post Synaptic Current for excitatory synapses (EPSC).

On the other hand, an amplifier in “current clamp mode” will not inject any current, it will simply measure changes in cell voltage. This will be termed as Excitatory Post Synaptic Potential (EPSP), or “field potential” when electrode is placed outside the cell and is

measuring changes in the electric field (Hill & Stephens, 2021).

Usually, the electrode is placed into a thin glass pipette filled with a solution similar to the cytoplasm of the cell which is going to be measured. If the pipette and the cell are close enough, the applying of negative pressure from inside the pipette will create a tight seal between the pipette and the cell membrane. This technique called patch clamp method (Sakmann & Neher, 1984) is very common for the measurement of synaptic responses.

1.4 Probability of release

There are two main factors that contribute to the size of synaptic responses: on the presynaptic side, the amount of neurotransmitter released; on the postsynaptic side, the number of receptors available is determinant.

The amount of neurotransmitter released depends at the same time on three elements: the number of vesicles readily available for release, the probability of release for each releasable vesicle (i.e., fusion efficiency) and the amount of neurotransmitters inside them. The vesicles storing neurotransmitter are not homogeneously distributed along the presynaptic terminal, they are separated into "pools". As mentioned previously, not all vesicles are in the primed state necessary for being released. Only those that are docked to the plasmalemma of the AZ, a group named as readily releasable pool (RRP), have the possibility of turn into the primed state. After being emptied, the RRP is replenished by vesicles that are recruited from the reserve pool thanks to downstream movement from one pool to another. A bigger RRP means more vesicle available for exocytosis, and thereby more neurotransmitter release after each action potential (Kaeser & Regehr, 2017; Thanawala & Regehr, 2013).

On the other hand, there is the probability of release, a term used to quantify the release of neurotransmitter from presynaptic terminals. In some reports, probability of release is meant to describe the probability with which a given synapse releases neurotransmitter following an action potential. And in others, the same term is meant to describe the probability for a readily releasable vesicle to undergo exocytosis. This can be confusing because most synapses have multiple readily releasable vesicles, meaning that the probability of release per synapse and per readily releasable vesicle differ greatly. To avoid confusion, the term used are *probability of release per synapse* or *probability of release per vesicle* depending on the concept we are trying to convey. In this research, the primarily interest is in probability of release per vesicle, which will be

denoted with p_v. There are evidences about different p_v*s* depending on the release site to which the vesicle is docked(Mahfooz et al., 2016; Taschenberger et al., 2016). Several factors can influence in the p_v of individual release sites, such as the distance to nearby VGCCs or the SNARE architecture at that moment (Chen et al., 2015; Rizo, 2018). The p_v can be modified using drugs like phorbol-esters or by changing the extracellular Ca^{2+} concentration. E.g., activation of Munc13 and Complexin increases p_v (Liu et al., 2021; Lou et al., 2008).

A higher amount of Ca^{2+} outside the cell will make that each time a VGCC gets open by an action potential, more ions flow inside the terminal due to the enhanced difference in concentration. Therefore, more Ca^{2+} will be available for SYT1 binding, increasing then the probabilities of a ready releasable vesicle to undergo exocytosis in each action potential. This works in the opposite way also. Less extracellular Ca^{2+} concentration will reduce the p_v because just a few ions enter the presynaptic terminal with each action potential. Lower Ca^{2+} entrance means less SYT1 binding, so lower probabilities of release. There are other p_v negative regulators besides low Ca^{2+}, like Mover or Synaptophysin (Körber et al., 2015; Raja et al., 2019).

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1.5 Synaptophysin

Synaptophysin 1, Synaptophysin 2, Synaptogyrin 1 and Synaptogyrin 3 constitute a family of proteins embedded in the membrane of vesicles, expressed widely throughout the animal kingdom (Fernández-Chacón & Südhof, 1999; Stenius et al., 1995). Synaptogyrin 2, or Cellugyrin, is a non-neuronal member of the Synaptophysin family (from this moment named as just Syp family) (Janz & Südhof, 1998). These proteins are the second most abundant in the vesicular membrane after VAMP2, with which they may form a complex. Even though the possible interaction side with VAMP2 is not well established, it is known that Syp family is embedded into the vesicle membrane through four transmembrane domains, leaving two loops inside the vesicle and one outside.

There is a debate about the role of Syp family. Nonetheless, recent studies showed that the members are likely negative regulators of exocytosis, acting downstream of docking and priming (Raja et al., 2019). Indeed, the deletion of the whole family turns synaptic responses stronger. The quadruple knockout mice (QKO) for the four proteins exhibit larger EPSCs than wildtype (WT) at a variety synapses under standard conditions. Unlike the differences observed between WT and QKO responses, no alteration in the RRP size of the QKO is evident.

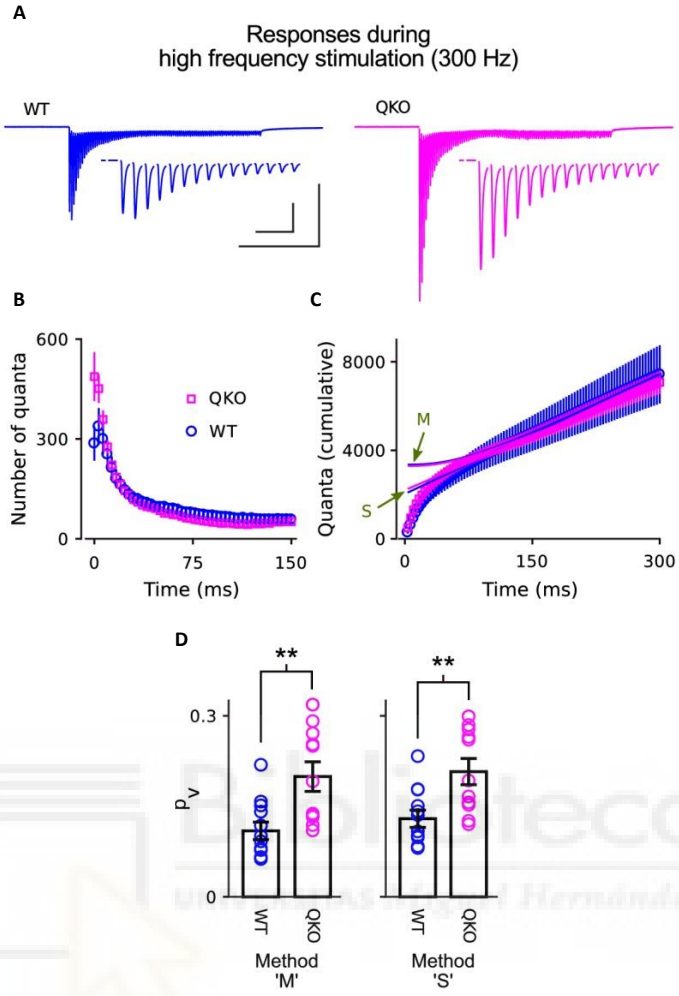


Figure 1 QKO synapses have an enhanced p_v. To resolve which of the parameters determining synaptic responses was altered, calyx of Held synapses were stimulated at 300Hz for 300ms (A) (Scale bars are 1 nA *vs* 100 ms (outer) and 1 nA *vs* 10 ms (inner) corresponding to the insets showing first 15 responses). The difference in synaptic strength disappeared quickly, by the 4th action potential (A-B), and there was no difference in the total number of quanta released during the first 150ms of the trains (C). The result suggests that the RRP content was not altered at QKO synapses because 150 ms is enough to nearly completely exhaust the RRP (Mahfooz et al., 2016). Cumulative amount of quanta released was measured by different methods. Line marked 'S' is calculated using the method in Schneggenburger et al., 1999, whereas lines marked 'M' is calculated using Eqn in Mahfooz et al. (2016). p_v is calculated by dividing the number of quanta released after isolated action potentials by the RRP content, and was approximately double at QKO synapses (D) Adapted from Raja et al.2019

As mentioned before, the number of vesicles that undergo exocytosis depend on the number and size of vesicles within the RRP and the p_v. A change in the number of receptors seems unlikely because of the Syp family presynaptic location. Therefore, the

bigger EPSCs in QKO must be caused by the first mentioned factors. In the paper from Raja et al., 2019, we can see how there is a difference in p_v in Schaffer collaterals and calyx of Held between WT and QKO animals. Dividing the total quanta released during high frequency trains by the isolated action potentials we obtained a difference in the p_v of the QKO mice synapses with respect to the WT. This is supported by an occlusion in Short-Term Plasticity, so an enhancement in QKO p_v seems the most logical explanation. However, this does not rule out the possibility of changes in the amount of neurotransmitter within the vesicles. Indeed, ongoing studies might show how larger vesicles in QKO would contribute to larger synaptic responses together with then enhanced p_v.

This native enhanced p_v of QKO mice will be used as tool during the present thesis due to the possibilities it opens about determining how it affects synaptic plasticity.

2. Synaptic plasticity

Plasticity, as noted above, refers to the changes in function a synapse experiences and the modifications on its response based on the different activity of the neurons involved. These changes can result in a stronger (potentiation) or a weaker (depression or depotentiation) synaptic response. Most of the modifications last from tens of milliseconds to a few minutes, which is referred to as Short-Term Plasticity (STP). Under certain conditions, the modifications in synaptic strength can last longer or even became permanent. In those cases, the term Long-Term Plasticity is used.

2.1 Short-term Plasticity

The changes attributed to STP can occur in most types of chemical synapses during repetitive use. These modifications can range from tiny differences in the synaptic response to even a 10-fold increase, depending on the type of synapse and plasticity event. Two apparently similar synapses can have completely different properties (Hempel et al., 2000).

Based on the direction in which the synaptic response size changes, STP events are classified as depression for lower responses, and short-term enhancement for potentiated ones. Inside this last term we can find specifically facilitation, potentiation and augmentation.

Quantal analysis has revealed that the variety of STP potentiation events have

a presynaptic origin. Concretely, modifications in the number of molecules of neurotransmitter released without altering postsynaptic sensitivity. Hence, these alterations are considered as a possible information-processing and computational tool before other synaptic network changes (Hennig, 2013; Zucker & Regehr, 2002).

Nevertheless, the mechanisms behind each STP event differ among them, requiring a more detailed explanation of each one.

2.1.1 Depression

The repetitive stimulation may turn some synapses into a decrease of its synaptic efficiency, in other cases an increase, and sometimes both are able to happen. This phenomenon is known as Short-Term Depression (STD) when synaptic efficiency gets reduced (Dodge & Rahamimoff, 1967). STD is commonly caused by a reduction in neurotransmitter release due to a lowering in the p_v or a shortage in readily releasable vesicles. If a synapse is continuously stimulated faster than the time it takes for the RRP to be replenished, responses will become depressed because there are not enough vesicles available for exocytosis. This depletion would be reached earlier in case a mechanism was lowering the replenishment of RRP(Schneggenburger et al., 2002; Stevens & Wang, 1995).

The translocation of VGCCs further from release sites or their inactivation by repetitive brief depolarizations leads to a lower Ca^{2+} entrance to the AZ. This consequently reduces the p_v as in conditions of low extracellular Ca^{2+} concentration. Other mechanisms that could explain STD include inactivation of release sites and postsynaptic receptor saturation/desensitization (Forsythe et al., 1998; Han et al., 2011; Neher & Sakaba, 2003).

2.1.2 Facilitation

Most synapses that include vesicles with a low p_v release more neurotransmitter if a second action potential arrives shortly after the first one in the presynaptic terminal (Katz & Miledi, 1968). This phenomenon, known as facilitation, depends on the presynaptic Ca^{2+}. As mentioned before, each time VGCCs open, Ca^{2+} ions flow into the cell, triggering vesicle exocytosis. Ca^{2+} buffers must remove Ca^{2+} ions rapidly from the AZ. But this clearance sometimes is not completed before the reopening of the channels

(which only takes a couple of ms) by the arrival of a new action potential.

The explanation for this event commonly involves residual Ca^{2+} not being cleared from the terminal, accumulating with the new influx, thereby enhancing p_v and subsequently getting larger synaptic responses. Hence:

$EPSC_1$ = (constant * Ca^{2+} influx)^n

$EPSC_2$ = (constant * Ca^{2+} influx + residual Ca^{2+})^n

Here, EPSC1 is the response to the first action potential, EPSC2 the response to the second one, and n is 4. The enhancement in the second response will be inversely proportional to the time separating both action potentials, disappearing once the Ca^{2+} has been completely cleared. However, this clearance time differs for each synapse due to distinct Ca^{2+} clearance mechanisms among synapses.

There are some synapses where this model cannot explain facilitation (Schneggenburger & Neher, 2005), opening the door to theories with other mechanisms. A second possibility explains facilitation with a high-affinity Ca^{2+} sensor. This sensor is not working during the first action potential, but the residual Ca^{2+} activates it, so it will coordinate with the main Ca^{2+} sensor during the second action potential. The high-affinity sensor is involved in triggering release at slow high-affinity Ca^{2+} binding sites, distinct from those active in normal cooperative release (Atluri & Regehr, 1996; Sippy et al., 2003). On the other hand, a third possibility implies a high-affinity Ca^{2+} buffer that binds rapidly Ca^{2+} ions, lowering p_v, but it is overwhelmed during the second action potential, recovering then p_v (Neher, 1998; Roberts, 1993). A fourth theory suggests that VGCCs are responsible for facilitation. This is supported by experiments demonstrating activity-dependent enhancement of Ca^{2+} currents during facilitation (González Inchauspe et al., 2004; Ishikawa et al., 2005), and proteins such calmodulin regulating VGCCs (Catterall & Few, 2008).

All these theories share two common elements: the significance of residual Ca^{2+} and a subsequent increment in p_v. Furthermore, in Raja et al, 2019 was shown how the enhanced p_v in QKO occluded facilitation.

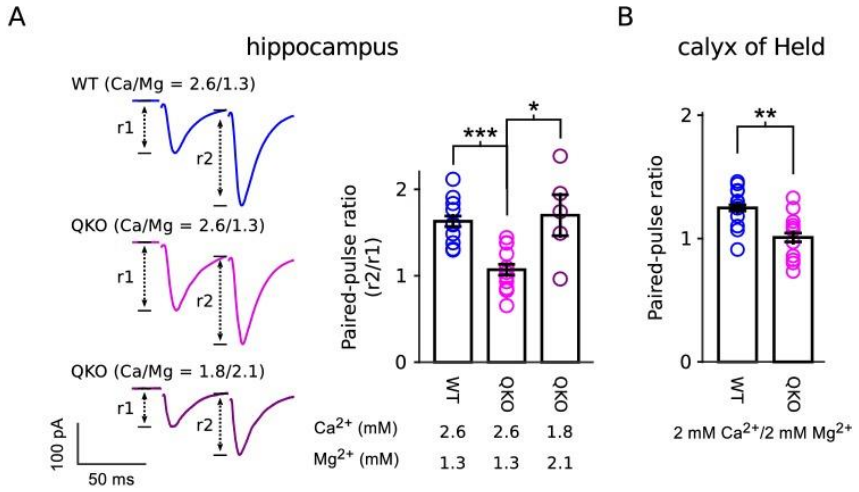


Figure 2 Facilitation occluded in QKO synapses Synaptic facilitation In Schaffer collaterals is occluded in QKO synapses compared to WT (A). Under the same Ca^{2+} conditions, WT synapses show bigger synaptic enhancement during paired pulses separated by 50 ms than QKO synapses. If Ca^{2+} is reduced, and subsequently the p_v, facilitation is recovered in QKO synapses. (B) Calyx of Held synapses also show occlusion during facilitation. Adapted from Raja et al.2019

Indeed, in the same research we showed how increases in p_v might interact among them independently of the origin of it. So, an increase in p_v by one mechanism such as knocking out Syp family or raising extracellular Ca^{2+} would occlude a possible posterior increase by facilitation.

2.1.3 Post-Tetanic Potentiation

Post-Tetanic Potentiation (PTP) is an enhancement of the synaptic strength caused by a sustained high-frequency stimulation (tetanic stimulation) (Barrett & Stevens, 1972; J. del Castillo & Katz, 1954; LLOYD, 1949). Like facilitation, it is exclusively presynaptic, and the induction protocols vary for each synapse, getting different potentiation levels. It can last from tens of seconds to several minutes, depending on the length of the tetanic stimulation, suggesting a direct relationship between both (Magleby, 1973). This tetanic stimulation will cause an accumulation of residual Ca^{2+} into the terminal due to repetitive action potentials arriving faster than the clearance.

The importance of this residual Ca^{2+} is a shared feature for all the theories behind

PTP regardless of the proposed model or the synapse. One mechanism includes the possibility that tetanic stimulation elevates residual Ca^{2+} by an increase in action potential evoked Ca^{2+} influx, thereby increasing p_v. In the calyx of Held, a Ca^{2+} decay that followed the decay of the potentiation has been observed (Habets & Borst, 2006; Korogod et al., 2007). Some protein cascades with slower kinetics than clearance have been proposed as PTP regulators.

The Ca^{2+} accumulated after tetanic stimulation follows two ways. One part is cleared rapidly out of the cell by Na^{+}/Ca^{2+} exchangers. The other is accumulated into the mitochondria. Once mitochondria are full, Ca^{2+} is released slowly, increasing p_v and generating the PTP. This is supported by experiments where blockade of Ca^{2+} entry to mitochondria prevents the late phase of Ca^{2+} decay and hence the PTP (D. Lee et al., 2007; Lou et al., 2008; Y. G. Tang & Zucker, 1997).

It has been suggested that an increase in RRP size in the calyx of Held could contribute to PTP, but studies show how the size of the pool remains intact (J. S. Lee et al., 2010). Instead, the fast-releasing component of the pool is increased and slow one is decreased. This change might be also interpreted as a modification in the distance of the vesicles to the VGCCs, which would change their p_v (Neher & Sakaba, 2008; Sakaba & Neher, 2001).

Several experiments involve Protein Kinase C (PKC) in PTP (Brager et al., 2003; Korogod et al., 2007). However, the elevated number of PKC isoforms makes difficult to discern its realrole in PTP.

Independent of these studies, most of the models point into a transient increase in the p_v as the final effector of the PTP synaptic response enhancement.

2.2 Long-Term Plasticity

As in STP, long term changes in plasticity affect synaptic responses. But there is a substantial difference; instead of occurring from milliseconds to minutes, long-term changes extend from hours to days, weeks or even months (Abraham, 2003). Similar to STP, strong or repetitive synaptic activity initiates Long-Term Plasticity, although in some synapses,there is a requirement for strong postsynaptic side activity as well.

Again, these plastic changes can cause both enhancement and depression of the synaptic response. Indeed, Long-Term Plasticity is basically comprised by two phenomena: Long-Term Potentiation (LTP) and Long-Term Depression (LTD), which

enhance or decrease the synaptic response respectively. However, according to the functionality of these modifications, theories are quite different from STP.

Since its discovery by Terje Lømo and Timothy Bliss in 1966, Long-Term Plasticity has been postulated as the mechanism for memory formation. The long duration of the event and the possibility of being evoked at single synapses make it a perfect candidate. A lack of understanding about how Long-Term Plasticity really works does not displace it from this role. Richard Morris and colleagues formalized a presumptive causal link between synaptic plasticity and memory:

"*Activity dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which plasticity is observed"* (Martin et al., 2000).

Thereafter, different brain areas and their subsequent synapses involved in different kinds of memory have been identified. Declarative memory, which can be recalled as verbal or nonverbal material (ideas, sound, images…), is located in midline diencephalic and medial temporal lobe structures. The skill-associated memory is denominated non-declarative, an umbrella term referring to multiple forms of memory that are not declarative (Squire & Zola-Morgan, 1988). The wide variety of non-declarative memory categories imply many brain structures, such as the basal ganglia, prefrontal cortex, amygdala, striatum, sensory association cortices and cerebellum (Knowlton et al., 1996; Squire, 2004; Squire & Dede, 2015).

Inside the temporal lobe, hippocampus is involved in spatial memory (O'Keefe & Dostrovsky, 1971). Besides, it also seems to work as a precursor for all declarative memories (Wixted & Squire, 2011) and recognition (Eichenbaum et al., 2007; Squire et al., 2007). Furthermore, since the case of Henry Molaison, hippocampus has been a focus for memory storing theories. Molaison went under a bitemporal lobectomy for treating his epilepsy, losing during the process 2/3 of the hippocampus, hippocampal gyrus and amygdala. This surgery cured the epilepsy, but the patient lost the ability of gaining new memories, although the old ones were intact (Squire, 2009). Since that moment, hippocampus remains as the most used region for memory studies, even before Long-Term Plasticity discovering.

Indeed, the first Long-Term Plasticity discovery made by Bliss and Lømo was in this region, and since then, multiple studies have been performed there. There are many characteristics that make the hippocampus a good memory storage, especially three. First, the cooperativity, by which a number of presynaptic fibers can be simultaneously

activated and 'cooperate' to elicit Long-Term Plasticity. Then, input-specificity ensures that once Long-Term Plasticity is activated in a single synapse, the rest of them are intact, providing ample space for memory storage. Additionally, there is the associativity, wherein weakly activated synapses are potentiated if a neighboring synapse is strongly stimulated, resulting in the potentiation of both synapses (Andersen et al., 1977; Kitajima & Hara, 1991; Lynch et al., 1977; Wörgötter et al., 2018).

Many differences have been discovered over the years in the mechanisms responsible for Long-Term Plasticity among distinct types of synapses within the brain, making difficult a general description of the phenomenon. For this reason and because it is the main studied brain area in this thesis, subsequent descriptions of LTP and LTD mechanisms will be explained according to Schaffer collateral synapses in the hippocampus.

2.2.1 Long-Term Potentiation

As mentioned earlier, Long-Term Plasticity, specifically Long-Term Potentiation (LTP), was discovered by Bliss and Lømo in the hippocampus, more precisely in the perforant path from the entorhinal cortex in rabbit (Bliss & Lømo, 1973). There are several forms of LTP depending on the hippocampal region and whether they are NMDA receptor-dependent or not (Grover & Teyler, 1992; Teyler et al., 1995). For example, in the dentate gyrus, there is an NMDAR-independent LTP (Hashimotodani et al., 2017), while in the Schaffer Collaterals, high-frequency stimulation induces LTP through the opening of NMDAR channels (Collingridge et al., 1983; Harris et al., 1984; Morris et al., 1986). Most research focuses on NMDAR-dependent LTP, as it serves as an appropriate model for associative learning. So, the upcoming description will be of this particular form of LTP.

An overview explanation of the molecular mechanisms underlying LTP would be that is the result of a postsynaptic cascade of events initiated by Ca^{2+} entering through the NMDAR channels (Granger & Nicoll, 2013; Lüscher & Malenka, 2012). In fact, a postsynaptic Ca^{2+} chelator can inhibit LTP, while the injection of postsynaptic Ca^{2+} enhances it (Crépel & Ben-Ari, 1996; Neveu & Zucker, 1996). The (2*R*)-amino-5-phosphonovaleric acid (APV), an NMDAR blocker, was key to this discovery, because it blocks LTP without altering AMPA responses (Morris et al., 1986).

The NMDAR is a non-selective cation channel, a tetramer formed by different subunits which needs glutamate and glycine (this last one present in the extracellular

environmentof the brain) to allow Na^+ and Ca^{2+} entrance (Furukawa et al., 2005), initiating thus LTP. However, canonical NMDARs, which are made up of two NR1 subunits and any two of four possible NR2 subunits, have the channel pore blocked by Mg^{2+} at resting membrane potential conditions (Kutsuwada et al., 1992; Monyer et al., 1992; Sugihara et al., 1992). This does not allow Ca^{2+} entrance to initiate the LTP, so the postsynaptic cell must be depolarized to displace Mg^{2+} blockade (Mayer et al., 1984; Nowak et al., 1984).

High-frequency stimulation of the presynaptic terminal leads to repetitive activation of AMPARs by glutamate, causing a significant influx of Na^+ into the postsynaptic cell. This influx depolarizes the cell and expels Mg^{2+} from the NMDARs pore (Lüscher & Malenka, 2012).

Alternatively, depolarization at lower frequencies can be induced in the laboratory by injecting current directly into a postsynaptic cell with a microelectrode. This process raises the resting potential of the membrane, facilitating Mg^{2+} displacement from the NMDARs (Gustafsson et al., 1987; Kampa et al., 2004; Malinow, 1991). Furthermore, it can be paired with presynaptic stimulation as part of a pairing protocol.

The binding of glutamate released from the presynaptic cell to the NMDARs, combined with the removal of Mg^{2+}, permits Ca^{2+} to enter the postsynaptic cell, initiating LTP. Both the high-frequency stimulation and the pairing protocol methods align with the fundamental principle of synaptic potentiation when pre- and postsynaptic elements are activated simultaneously.

2.2.1.1 Early-Long-Term Plasticity

The duration of the synaptic potentiation resulting from LTP can vary based on the induction method, and consequently, the events initiated by it. Potentiation goes through two consecutive phases: early LTP (E-LTP) and late LTP (L-LTP) (Frey et al., 1993).

The mechanism for LTP expression has been since many years under debate. A substantial number of evidences suggests that the enhancement of synaptic responses in E-LTP occurs through the increases in both the number and single-channel conductance of AMPARs. This would occur at the postsynaptic density (PSD), a protein-dense area attached to the postsynaptic membrane, opposite to the presynaptic terminal (Shi et al., 1999). The receptor amount increment comes from AMPARs located in perisynaptic zones moved to the PSD together with intracellular

vesicles inserting more receptors into the membrane by exocytosis (Makino & Malinow, 2009). SYT1 and 7, together with VAMP2, SNAP-47 and Syntaxin-3 are implicated in the exocytosis of AMPARs (Jurado et al., 2013; Wu et al., 2017). However, this AMPAR trafficking works in the opposite way also, so to achieve the potentiation, receptors must be retained in the PSD.

An initial increase in intracellular Ca^{2+} activates many enzymes, most of them kinases such as PKC and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Racioppi & Means, 2012). Individual CaMKII molecules are made up of 10-12 subunits, each one with catalytic and autophosphorylation domains (Hudmon & Schulman, 2002). Ca^{2+} binds to calmodulin (CaM), and together they release the regulator segment of CaMKII, which acts as an autoinhibitory subunit. This allows CaMKII autophosphorylation, keeping the enzyme active even after the Ca^{2+} concentration decreases (Chao et al., 2010). Phosphorylated CaMKII binds to the NR2B subunit of NMDARs and phosphorylates proteins in PSD (Lisman, 1985; Lisman & Goldring, 1988). One of the main targets of CaMKII after NR2B binding are transmembrane AMPAR-regulatory proteins (TARPS) (Kristensen et al., 2011; H. K. Lee et al., 2000).

Phosphorylated TARPs bind to protein PSD95 via the PDZ domain, maintaining AMPARs in the PSD (Sumioka et al., 2010, 2011). The AMPARs permanency at the PSD will enlarge synaptic responses. This potentiation, which can last up to two hours, is called E-LTP.

2.2.1.2 Late-Long-Term Potentiation

Repeated stimuli and higher frequency protocols can prolong synaptic response enhancement. A more continuous influx of Ca^{2+} entering through NMDARs activates additional protein cascades after E-LTP events occur, resulting in changes in gene expression. These processes cause structural modifications in the dendrites via newly synthetized proteins,which extend potentiation from hours to days. This is referred to as L-LTP (Abel et al., 1997; Frey et al., 1993; Kandel, 2001).

The limit where postsynaptic Ca^{2+} influx activates L-LTP responsible elements besides E-LTP ones is not well established. However, it is known that one of the main effectors is cyclic adenosine monophosphate (cAMP). Activation of the cAMP response element- binding (CREB) transcription factor seems to be required for L-LTP in Schaffer collateral synapses (Huang et al., 1994). It can be achieved in different ways (Alberini, 2009), but the most studied is the one via second messenger cAMP.

Ca^{2+}/CaM sensitive adenyl cyclase activation by the Ca^{2+}/CaM complex initiates cAMP generation (Elliot et al., 1989). The binding of cAMP to the regulatory subunits of Protein Kinase A (PKA) releases its catalytic subunits, which will phosphorylate and activate CREB in the nucleus (Nguyen & Woo, 2003). Adenyl cyclase can also be activated by some GPCRs (W. J. Tang & Gilman, 1991). CREB target genes include the proteins C-fos, activity regulated cytoskeleton-associated protein (Arc), and brain-derived neurotrophic factor (BDNF) (Finkbeiner et al., 1997; Kawashima et al., 2009; Sheng et al., 1991), which have all been implicated in cell remodeling and proliferation.

There are other proteins involved in L-LTP that are not thought to depend on CREB. Cytoplasmic polyadenylation element binding (CPEB) is a prion-like protein that regulates activation of dormant mRNAs during LTP (Si et al., 2003, 2010). One of the most studied proteins nowadays is PKMζ due to its role in preventing AMPAR lateral diffusion to maintain LTP (Si et al., 2003; Yu et al., 2017)

The molecular mechanisms behind LTP are still far from being completely understood, and the number of proteins implicated grows constantly. However, a tilt in the balance of exo-endocytosis of AMPARs seems to be necessary for E-LTP, and the same about de novo postsynaptic protein synthesis for L-LTP.

2.2.2 Long-Term Depression

As its name indicates, this form of plasticity consists of a long-lasting decrease in the synaptic response. The first reference come from Lynch et al., 1977, who described a depotentiation in some Schaffer collateral neurons during an LTP experiment. The following year, the same group described how 100 low-frequency pulses at the same synapse could decrease the synaptic response (Dunwiddie & Lynch, 1978).

It is important to point out that, even though a long-lasting decrease in the synaptic response size may sound like a pathology, most Schaffer collateral synapses can undergo LTD at any moment (Dudek & Bear, 1992). If synapses only had the ability to get potentiated, the brain would reach a state of full capacity, making it difficult to encode new information. This is one of the main arguments behind the existence of LTD. It allows synaptic modifications in strength so selective weakening of some synapses make the potentiation of others useful(Ito et al., 2014).

The kind of responsible mechanisms for the synaptic response decreasing are the opposite of LTP ones. LTD molecular mechanism is mainly based on phosphatases instead of kinases. The most studied are phosphatase 1 (PP1) and phosphatase 2B

(PP2B) or calcineurin (Mulkey et al., 1993).

Again, like in LTP, the role of NMDARs seems to be essential. Depolarization displaces Mg^{2+} allowing Ca^{2+} to enter the postsynaptic cell, but in this case, there must be a slow increase in the intracellular concentration of the ion instead of a fast increment (Dudek & Bear, 1992). This smooth increase in Ca^{2+} activates PP1 and PP2B, which dephosphorylate AMPAR GluA1 subunit, reducing its conductance and promoting its endocytosis (Fox et al., 2007; Malenka & Bear, 2004). As result of the deficiency in AMPAR availability and conductance, synaptic responses are reduced in size.

An LTD based on the inactivation of group I metabotropic glutamate receptors and independent from NMDA have been observed in some studies (Fitzjohn et al., 1999; Kemp & Bashir, 1997; Palmer et al., 1997). However, the mechanisms behind this are not clear yet.

2.2.3 Presynaptic Long-Term Plasticity

Despite of mentioned before, Long-Term Plasticity is not just expressed postsynaptically in Schaffer Collaterals. Along the years, presynaptic modifications in Long-Term Plasticity have been associated to hippocampal mossy fibers, where evidence showing changes in neurotransmitter release have imposed over postsynaptic remodeling (P. E. Castillo, 2012). In the opposite way, many of the studies in Schaffer collaterals point to postsynaptic mechanisms as responsible for Long-Term Plasticity changes (Baltaci et al., 2019). However, things are not so clear.

Following the explanations about Long-Term Plasticity in Schaffer collaterals, we can find mechanisms altering presynaptic proteins and characteristics. Rabs are GTP binding-proteins implicated in the membrane trafficking (Schlüter et al., 2004). Concretely, Rab3 subfamily and one of its effectors, RIM1, all substrates of presynaptic PKA, have a role in neurotransmitter exocytosis. It has been observed how the deletion of the whole subfamily has consequences in the maintaining of NMDAR-dependent LTP. Although induction of LTP is normal in these KOs, the L-LTP is reduced respect to WT synapses (Huang et al., 2005). Furthermore, the release of neurotransmitter is enhanced during late phases of L-LTP (Bayazitov et al., 2007). Altogether, these researches suggest a role for the presynaptic terminal in L-LTP consolidation which could be through modifications in presynaptic vesicle proteins, mitochondrial changes or even redistribution of VGCCs (Nanou et al., 2016; H. L. Smith et al., 2016).

Indeed, so as LTP and LTD seem to share alterations in AMPARs amount and distribution for their expression, neurotransmitter release may also be modified during LTD in Schaffer Collaterals. Some studies show how neurotransmitter release is reduced at late states of some LTD forms (Andrade-Talavera et al., 2016; Sanderson et al., 2022). The responsible behind this could be calcineurin, which not only dephosphorylates proteins on the postsynaptic side. Synapsin I is one of the main presynaptic targets of calcineurin, and its dephosphorylation causes reduction in the size of the RRP (Bykhovskaia, 2011; Hilfiker et al., 1999). This presynaptic calcineurin activation can be due to retrograde intercellular diffusible messengers released during LTD, such as nitric oxide or arachidonic acid (Bolshakov & Siegelbaum, 1995; Stanton et al., 2003; Zhang et al., 2006). Together with the decrease in neurotransmitter release, a reduction in the number of presynaptic boutons has been observed during LTD (Becker et al., 2008).

These presynaptic perspectives of the NMDAR-dependent Long-Term Plasticity in Schaffer collaterals are always under debate competing with the postsynaptic evidence. The heterogeneity and diversity in the mechanisms behind this form of Long-Term Plasticity is just reminding the complexity of the event and the needed of research taking all possibilities in account.

3. Comparative models

There are so many types of synapses that generalizing without accounting for exceptions is risky. Aside from differences in morphology and functionality, there are also physiological variations. Not every synapse can undergo all types of plasticity modifications, and certainly those that are able to undergo them do not do so in the same way. Due to this, making assumptions about synaptic changes requires comparative studies to check the reproducibility of the events. In this thesis, synapses used for the research have been chosen based on their history in the field of plasticity and well-described bibliography.

3.1 Schaffer collaterals

Inside the hippocampus, forming the last step of the trisynaptic loop, along with the perforant path and the mossy fibers, are the Schaffer collaterals. This synapse, discovered by Károly Schaffer, facilitates communication between the axons coming from the contralateral Cornus Ammonis (CA) 3 and the dendrites of the pyramidal CA1

neurons. It is located specifically in the hippocampus area known as *Stratum radiatum* (Abel et al., 1997).

Schaffer collaterals are one of the most studied glutamatergic synapses, serving as the primary model for electrophysiological Long-Term Plasticity studies (Andersen et al., 2006). It is known that they can exhibit all forms of plasticity, both Short and Long-Term, as discussed previously. As the major excitatory input to CA1, they are also a good candidate for molecular synaptic dynamics. Over the years, Schaffer collaterals have been implicated in studies related to plasticity, memory, and placement. All these factors make Schaffer collaterals an ideal candidate for plasticity research.

3.2 Calyx of Held

Discovered by German Hans von Held (Held, 1893), this giant synapse in the auditory pathway is commonly used in comparative studies. From the globular bushy cells of the ventral cochlear nucleus (VCN) emerge single large terminals that go to the cell body of the principal neurons in the medial nucleus of the trapezoid body (MNTB) (Borst & Soria Van Hoeve, 2012; Rusu & Borst, 2011; Schneggenburger & Forsythe, 2006). Axons between 2-3 µm diameter cross the midline of the brainstem to end in the calyx-type nerve terminal of the contralateral MNTB (P. H. Smith et al., 1991). Despite there are other different inputs received by the MNTB (Hoffpauir et al., 2006; Rodríguez-Contreeas et al., 2006; P. H. Smith et al., 1991), Calyces for Held are monosynaptic.

In both mice and rats, around ~50% of the soma of the MNTB neuron is covered by the calyx of Held (Sätzler et al., 2002; Schneggenburger & Forsythe, 2006). Indeed, calyces are so large that they have between 300 to 700 AZs, even though those single zones have an estimated range of readily releasable vesicles not so different from hippocampal neurons (Rizzoli & Betz, 2005). Action potentials are faster and briefer (Taschenberger & Von Gersdorff, 2000) because the speed of this synapse is higher than normal ones (Borst & Soria Van Hoeve, 2012). This results in less opening of VGCCs, so p_v is higher to compensate (Iwasaki & Takahashi, 2001). On the other hand, the speed of the action potentials allows the synapse to support stimulation trains of 300 Hz frequency, ten times more than usual.

This special morphology and structural function guarantee a high-fidelity transmission of acoustic information for further processing by the auditory circuits (Trussell, 1999). The monosynaptic origin of calyx of Held is perfect for input-specific plasticity events. Also, the calyx of Held preparation allows electrophysiological analysis

of pre and postsynaptic compartments simultaneously, thanks to the possibility of patching both terminals at the same time.

All these characteristics make the calyx of Held a suitable candidate for studying synaptic transmission and plasticity, further solidifying its status as one of the most used models for comparative studies in the field.

Objectives

The research by Janz et al., 1999 into synaptic transmission at synapses from Synaptophysin 1 and Synaptogyrin 1 double knockout (KO) mice will be the inspiration for studying the Long-Term Plasticity of the QKO mice Schaffer collaterals. Although previous results in double KO already shown deficiencies in LTP, this has not been tested yet on this new genotype. Furthermore, no definite explanation was given for double KO phenotype. Therefore, the initial purpose of this thesis will follow the characterization of Syp family synaptic plasticity and how their influence in p_v can affect this.

This objective will go along with the idea of contributing to the finding of the mechanisms behind Long-Term Plasticity in Schaffer collaterals, which has been a debate since many years ago. The native enhanced p_v of QKO might be an explanation behind the phenotype of the double KO.

Whether the QKO synapses show differences in Long-Term Plasticity respect to the WT or not, the occlusion shown in QKO paired pulse facilitation (Raja et al, 2019) open a new question: Is the occlusion phenotype ubiquitous for the whole plasticity events? More insight about different forms of plasticity will be necessary for answering this last question. So, the objectives of the present thesis will be:

1. To determine a possible role of Syp family in Long-Term Plasticity.
2. To test if p_v is modified during Long-Term Plasticity thanks to the increased p_v observed in QKO mice.
3. To compare between PTP and LTP events to go deeper into the possibility of common mechanisms for Short- and Long-Term Plasticity.

Materials & Methods

1. Animals

Mice were selected for this study because QKO mice were already available in the laboratory. Animals were housed in groups of four to five per cage, provided with ad libitum access to food and water, and kept in a temperature-controlled and ventilated environment with a 12-hour dark/light cycle at the SEA (Spanish for Experimental Animal Services) vivarium of the Miguel Hernández University. All procedures adhered to European and Spanish regulations (2010/63/UE; RD 53/2013) and were approved by the Ethical Committee of the Generalitat Valenciana.

Knockout and WT control mice were obtained from the same cross. They underwent breeding for three or four generations by crossing a germline CRE-expressing line (Jackson Labs catalog number 008454) with Jackson line 008415. This line carries targeted KOs of Synaptophysin 1 and Synaptogyrin 1 genes, as well as floxed conditional mutations of Synaptophysin 2 and Synaptogyrin 3. The CRE transgene was eliminated during the breeding process and the WT animals were kept for a control line.

2. Solutions

2.1 Extracellular

The Artificial cerebrospinal fluid (ACSF) extracellular solution used for calyx of Held experiments was (in mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 2 $MgCl_2$, 2 $CaCl_2$, 0.4 L-ascorbic acid, 3 myo-inositol, 2 sodium pyruvate. Osmolarity was closed to ~310 mOsm and pH of 7.4 after bubbling with carbogen.

For hippocampus, ASCF composition was (in mM): 120 NaCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 3.5 KCl, 10 Glucose, 0.05 picrotoxin. The $CaCl_2$ and $MgCl_2$ concentration in ACSF varied to modify the p_v according to needs of the experiment. Both were added after previous bubbling of the ACSF with carbogen (95% O_2 and 5% CO_2) for at least 15 min to avoid calcium precipitation. The pH and osmolarity after bubbling with carbogen are 7.4 and ~300 mOsm respectively.

During STP experiments, the NMDA antagonist DL-APV was added to the ACSF with a final concentration of 50 µM. This avoids any possible potentiation in the responses due to spontaneous opening of NMDARs. In some LTP experiment it was added as control or for checking the residual potentiation of Ca^{2+} concentration change.

Glycine was added to the ACSF in all the Long-Term Plasticity experiments to ensure NMDAR opening with a final concentration of 20 µM.

2.2 Intracellular

For whole cell patch clamp hippocampus and calyx of Held recordings the intracellular solution used was (in mM) 130 Cs-gluconate, 10 CsCl, 5 Sodium phosphocreatine, 10 HEPES, 10 TEA-Cl (Tetraehtylammonioum chloride), 5 EGTA, 4 MgATP and 0.3 LiGTP; pH 7.2 and 290-300 mOsm.

3. Acute slice preparation

3.1 Hippocampal slices

For hippocampus experiments, the preparation used were brain slices from 12-16 days or 6-8 weeks old QKO and WT mice, according to the kind of experiment. All procedures were conducted in accordance with European and Spanish regulations (2010/63/UE; RD 53/2013) and were approved by the Ethical Committee of the Generalitat Valenciana (2022/VSC/PEA/0255).The animal was sacrificed by decapitation, head was placed into partially frozen ACSF-sucrose solution (in mM, 230 Sucrose, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 2.5 KCl, 2.6 $MgCl_2$ and 1.3 $CaCl_2$) previously bubbled with carbogen during at least 15 minutes, to decrease metabolic processes and neuronal activity. After that, the head was placed ventrally on a paper surface and clamped with forceps while skin was removed from the dorsal part by scissors cutting with a posterior-anterior cut. This allows the access to the skull. The same kind of cutting was performed on the skull adding lateral scission at bregma and lambda points, to later opening it carefully with forceps. Brain was removed introducing a spatula down the brain from the olfactory bulb and lifting it gently, to later cut the brainstem and extract the brain. About 1/6 of it was removed with a blade, from the anterior part in a 30º angle, and brain was vertically fixed with superglue (Loctite 404) at the base of the slicing chamber platform with the ventral surface oriented to the vibratome blade.

Slicing chamber was filled with partially frozen ACSF-sucrose solution until cover completely the brain. The slicing chamber was surrounded by ice to preserve ACSF-sucrose temperature below 5ºC and avoid therefore the increase of metabolic and neuronal activity. Vibratome Leica VT1200S settled with horizontal vibration at 80 Hz, 0.8 mm/s speed and a blade angle of 15º was used to obtain 400 µm brain slices while

chamber was bubbled with carbogen. Slices without hippocampal sections were discarded. The remnant ones were submerged in ACSF-sucrose solution into a Petri dish where CA3 section was removed by cutting the region. Slice recovery for 6-8 weeks old mice was done in 34ºC ACSF continuously bubbled with carbogen for 45-60 min and later to RT until use; 12-16 days old mice recovery was done directly in ACSF at room temperature (RT) (22-24ºC).

3.2 Calyx of Held preparations

For calyx of Held preparation, 14-19 days old QKO and WT mice were used. Head was dropped directly after decapitation into partially frozen modified sucrose-ACSF-calyx of Held solution (in mM, 85 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 75 sucrose, 0.5 $CaCl_2$, 7 $MgCl_2$, 0.4 L-ascorbic acid, 3 myo-inositol, 2 sodium pyruvate). Skin from the dorsal part was removed and skull opened with scissors and forceps in a similar way to hippocampus slicing. After brain lifting with a spatula, spinal cord was cut 0.5 cm below medulla oblongata, trying to let intact brainstem. Then brain was moved away from the skull. While brain was submerged in partially frozen sucrose-ACSF-calyx of Held solution, pia mater and arachnoid mater were removed with fine forceps under a microscope without touching olivary nuclei. After that, cerebrum was separated from cerebellum and brainstem with a proximately 45º angle cut. The remaining brainstem was placed with superglue at the slicing platform with the olivary nuclei facing the vibratome blade in a ~15º angle. Slice chamber was filled with partially frozen sucrose-ACSF-calyx of Held solution and bubbled with carbogen during the slicing process. Chamber was surrounded with ice to avoid temperature raising. Vibratome was settled at with horizontal vibration at 80 Hz, 0.8 mm/s speed and a blade angle of 30º was stablished to obtain 200 µm brain slices. Taking the middle point between superior and inferior olivary nuclei as reference, 3 slices before and 3 slices after this point were saved. Checking of the presence of antero-VCN axons under the microscope was used as extra control reference. Slices were moved to 34ºC ACSF-calyx of Held for 45-60 min of recovery and changed to RT until 7 hours later.

4. Experimental design

4.1 Electrophysiological Setup

The recordings were done in a submerged chamber full of continuously bubbled with carbogen ACSF transferred by a system of tubes and a peristaltic pump (RP1,

DYNAMAX, San Diego, USA) at RT. The slices were transferred there after incubation and kept under platinum harp grid with nylon wires. The chamber was situated over a microscope (BX51W1, Olympus, Japan) with an infrared differential interference contrast filter system. Slice observation was performed by a 4x air objective for extracellular recordings and a 40x water immersion objective (LUMPlan FI/IR, Olympus) for whole cell recordings, both connected to a CCD camera (KP-M2AP, Hitachi). Live visualization software was the Linux based *tvtime*. Manipulators for pipette placement were located laterally to the chamber, with two sets for stimulation and another two for recording, allowing double independent recording for extracellular recordings.

Recording pipettes for calyx of Held were made from borosilicate glass filaments, with an inner diameter of 0.86 mm and an outer one of 1.5 mm (GC150F-10, Harvard apparatus, UK). These pipettes were pulled by horizontal puller (Sutter instrument model p-97) with a resistance between 1.2-2.5 MΩ.

In the case of hippocampus experiments, borosilicate calibrated micropipettes of 100 µl (Drummond scientific company 2-00-100) were used and pulled using a two-stage vertical puller (PP-830 Narishige). For whole cell recordings, resistance pipette was set between 2.5-3.5 MΩ, while for extracellular recordings tip size was ~5 µm.

4.2 Electrophysiological recordings

All the experiments were performed at RT controlled by an in-line heater/cooler (Warner instruments, 64-0353). Amplifiers (Warner Patch Clamp PC-505B and Axon instruments Axopatch200B) and stimulators (A-M systems isolated pulse stimulator Model 2100 and Cibertec CS-420) were used indistinctly for whole cell and extracellular recordings experiments.

4.2.1 Whole cell recordings

4.2.1.1 Calyx of Held

For whole cell recording experiments, several trials were repeated in individual preparations for subsequent analysis. Individual MNTB neurons were patched in whole cell mode, and only cells with < 20 M series resistance allowing an electronic compensation of at least 80% were kept, with a holding voltage of -70 mV and no leak subtraction. Changes of no more than 30% in the series resistance were used as acceptance criteria among individual trials.

Calyx of Held presynaptic axons from the VCN were stimulated by a bipolar tungsten electrode, spanning the afferent fiber tract of the MNTB. As additional control, only cells responding to every action potential in high frequency stimulation trains were used.

Due to calyx EPSCs reaching ~20 nA, 1 mM kynurenic acid (KYN), a glutamate receptor antagonist, was added to the extracellular solution to reduce EPSCs magnitude and prevent receptor desensitization and saturation (Habets & Borst, 2007; Wesseling & Lo, 2002). 1 mM KYN decreases synaptic responses by ~85% in slope and current integral terms. APV was also added to avoid any NMDA possible response. ACSF used was specific for calyx slices with a Ca^{2+}/Mg^{2+} concentration of 2/2 mM.

4.2.1.2 Schaffer collaterals

For whole cell recording experiments, several trials were repeated in individual preparations for subsequent analysis. Individual CA1 pyramidal neurons were patched in whole cell mode, and only cells with < 20 MΩ series resistance were kept. Voltage was held at nominally -70 mV, without correcting for the liquid junction potential. Changes of no more than 30% in the series resistance were used as acceptance criteria among individual trials.

The CA3 was removed to prevent multisynaptic excitatory feedback responses caused by activation of this area by the monopolar stimulation electrode. This avoids subsequent interaction of CA3 with CA1 via *stratum oriens*. Monopolar stimulating electrode was placed in Schaffer collateral axons. Picrotoxin was added to the ACSF during the recordings to reduce GABA activity, and APV was used to block NMDA responses in experiments with no LTP induction.

4.2.2 Extracellular cell recordings

Extracellular recordings in current clamp mode were performed with the recording electrode placed in *stratum radiatum*. The limit of this area with the stratum *lacunosum moleculare* was the placement for the monopolar stimulating electrode. Both electrodes were positioned separately for at least 100 µm, ensuring a distance enough to get a field EPSP (fEPSP) in which fiber volley could be well appreciated. The stimulation intensity was adjusted to a third part of the maximum synaptic response.

5. Analysis

Software for recording experiments was designed by the lab written in C++. Data results were analyzed with software made by our own in MATLAB.

Results

1. Long-Term Potentiation is occluded by enhanced p_v

While the QKO mice synapses used in this research and previous from our lab were still being fully characterized, the role of some members of the Syp family in LTP had been previously studied. A double KO for Synaptophysin I and Synaptogyrin I was already described by Janz and Südhof (Janz et al., 1999). Their results showed how LTP was impaired in these mice, but they do not provide a solid reason behind the phenotype.

These experiments were performed in Schaffer collaterals, a synapse where historically LTP has been explained by pre- and postsynaptic perspectives, or even by both, but without reaching consensus. On the other hand, our previous research had been focused on STP events, which are presynaptic. The open debate about the mechanism behind LTP, together with the needed of characterizing our new QKO compared with the double KO mentioned above, guide us to the experiments described below.

We utilized the enhanced p_v in QKO mice to investigate its potential role in the mechanism underlying LTP and to delve deeper into the ongoing debate surrounding the origin of this mechanism.

1.1 LTP runs down more quickly at QKO synapses under standard conditions, but not when p_v is lowered by lowering extracellular Ca^{2+}

To test QKO LTP levels, experiments were conducted by measuring extracellular field recordings in hippocampus *ex vivo* slices, as in the Janz and Südhof article. The extracellular Ca^{2+}/Mg^{2+} concentration in ACSF was set at 2 mM for both, without APV and with the addition of 20 µM glycine to ensure NMDAR channels opening. The pipette placement was as explained in Material & Methods.

Before the induction protocol, we waited for a baseline of at least 20 minutes without alterations of more than 5% in the mean response size. After tetanic stimulation, responses were recorded for at least 60 minutes. Responses during baseline stimulation and after tetanus were recorded at 0.05 Hz to ensure the full replenishment of RRP in QKO. Experiments were conducted blindly for the genotype of WT and QKO.

The LTP induction protocol adopted involved two 1-second-long trains of 100 Hz stimulation, separated with a 20-second rest interval. This protocol, adopted to align with the previous analysis double KO synapses, revealed a persistent decline in LTP at QKO synapses (Figure 3). This result validates the conclusions drawn in the preceding study by Janz. However, unlike the sustained reduction in LTP noted for 1 hour post-induction in the previous study, we observed a gradual decline in fEPSPs at QKO synapses over time (Figure 3.C).

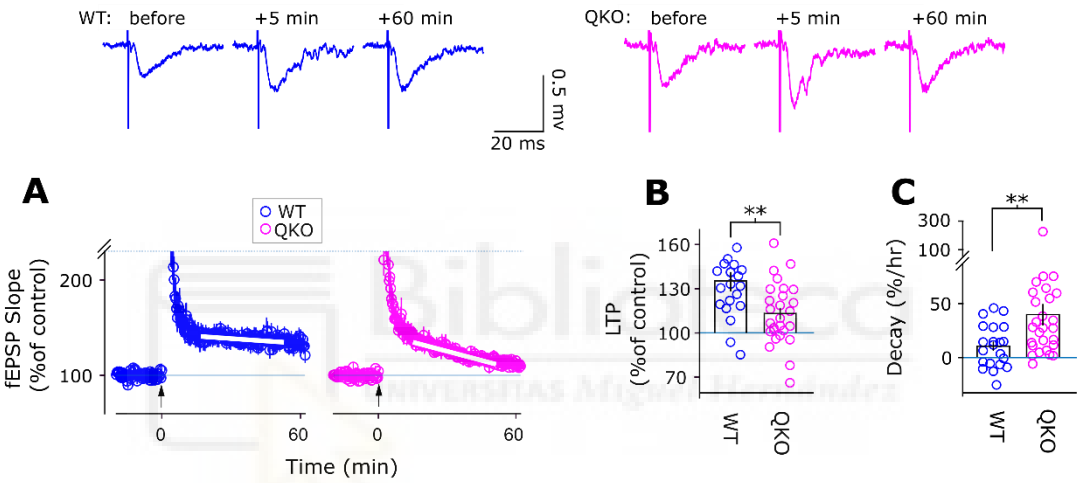


Figure 3 LTP decays faster in QKO. (A) 2 trains of 1s at 100 Hz separated by 20 seconds (arrow) elicit potentiation that is stable over one hour at WT synapses but that is lost with time at QKO synapses. Note that the y-axes are truncated, but the full range can be seen in Figure 4. (B). LTP was quantified at 51 - 60 after induction respect to the mean baseline (WT, 135 ± 7% n = 21, mice = 15; QKO, 113 ± 4% n = 27, mice = 19) (** signifies $p < 0.01$, rank sum). (C) Quantification of decay between minute 15 and 50 after induction (WT 11 ± 4%, QKO 40 ± 10%), matching the white lines in (A).

Notably, even the initial phase of potentiation, observed immediately after induction, appears to be attenuated at QKO synapses (Figure 4).

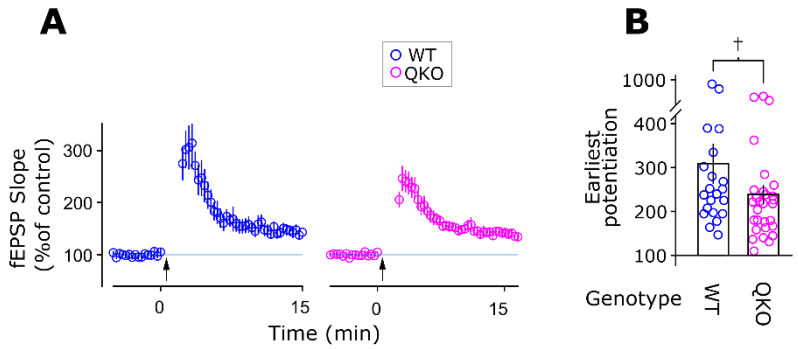


Figure 4 Earliest component of potentiation is probably reduced at QKO synapses. These results are from the same experiment as in Figure 3, except here the x-axis is truncated and the y-axis is not, to highlight the full early component of potentiation. The earliest potentiation for the first three data points immediately following induction was estimated respect to the mean baseline (WT 310 ± 47%, QKO 239 ± 22%) († signifies p = 0.057, rank sum).

1.2 Reducing extracellular Ca^{2+} rescues LTP

Addressing the deficit in LTP observed at QKO synapses, we hypothesized that a lower p_v might resolve the impairment as in STP deficits from Raja et al. 2019. To test this, we reduced extracellular Ca^{2+} from 2 mM to 1.2 in QKO slices, which likely decreased the p_v by lowering the amount of Ca^{2+} admitted into presynaptic terminals following individual action potentials. This concentration is closer to the physiological levels, which slightly superior to 1 mM (Ding et al., 2016; Forsberg et al., 2019).

Lowering Ca^{2+} successfully rectified the issue (Figure 5). Under these modified conditions, the sustained LTP for 1-hour post-induction at QKO synapses reached 139 ± 7% (n = 14) compared to the pre-induction baseline, which is similar to the value at WT synapses seen when Ca^{2+} was 2 mM (135 ± 7%; n = 21).

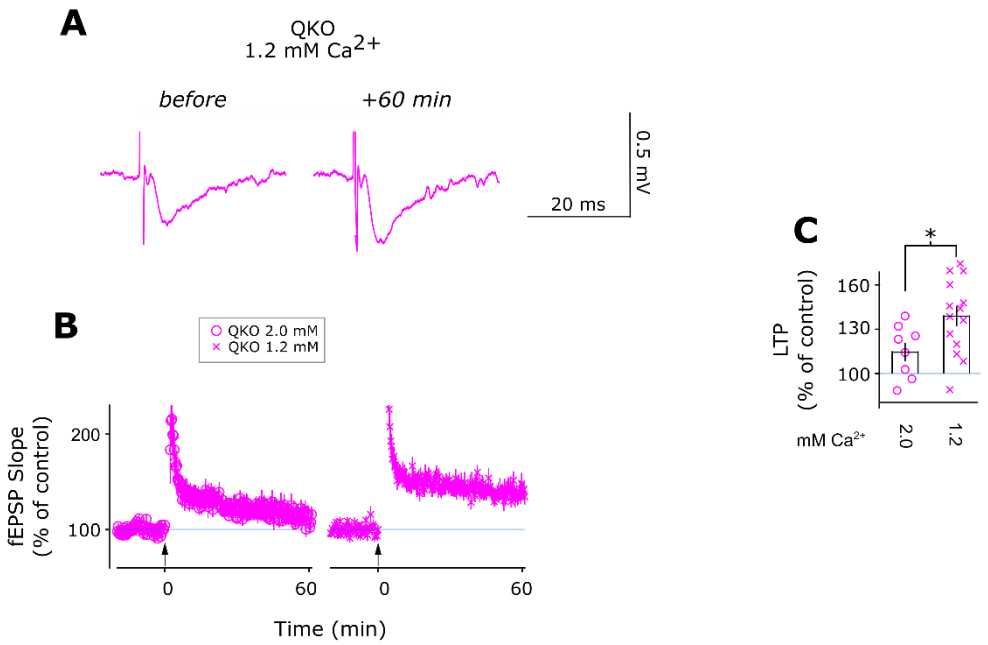


Figure 5 LTP is rescued by low Ca^{2+} in QKO. LTP rescue at QKO synapses when extracellular Ca^{2+} is 1.2 mM (n = 8, mice = 6) compared to 2 mM (n = 14, mice = 11). LTP induction and other experimental details match Figure 4, except the experimenter was not blind to experimental variables (* signifies $p < 0.05$, rank sum, $n \geq 4$). Experimental variables were interleaved. These data were gathered by Juan José Rodríguez Gotor and Doris Santiago and are included here by permission.

1.3 L-LTP runs down at QKO

LTP induction can be achieved using a variety of protocols, and the details affect the amplitude and persistence of the potentiation. We thought that a more intense induction protocol might reverse the decay in the potentiation seen at QKO synapses when extracellular Ca^{2+} was 2 mM. To test this, we instead induced with four 100 Hz frequency trains of 1 s each, separated by 5 min. This protocol was chosen because it is widely used to induce long-lasting LTP (L-LTP), which persists for more than 3 hours after induction (Kandel, 2001). . Experiments were conducted blindly for the genotype of WT and QKO at 2mM Ca^{2+}.

However, even with the intensified induction protocol, potentiation at QKO synapses decayed away almost completely within 2 hours whereas potentiation was maintained at WT synapses (Figure 6.A). Conforming with the results in Figure 5, the deficit was remedied once more by reducing extracellular Ca^{2+} to 1.2 mM (Figure 6.B).

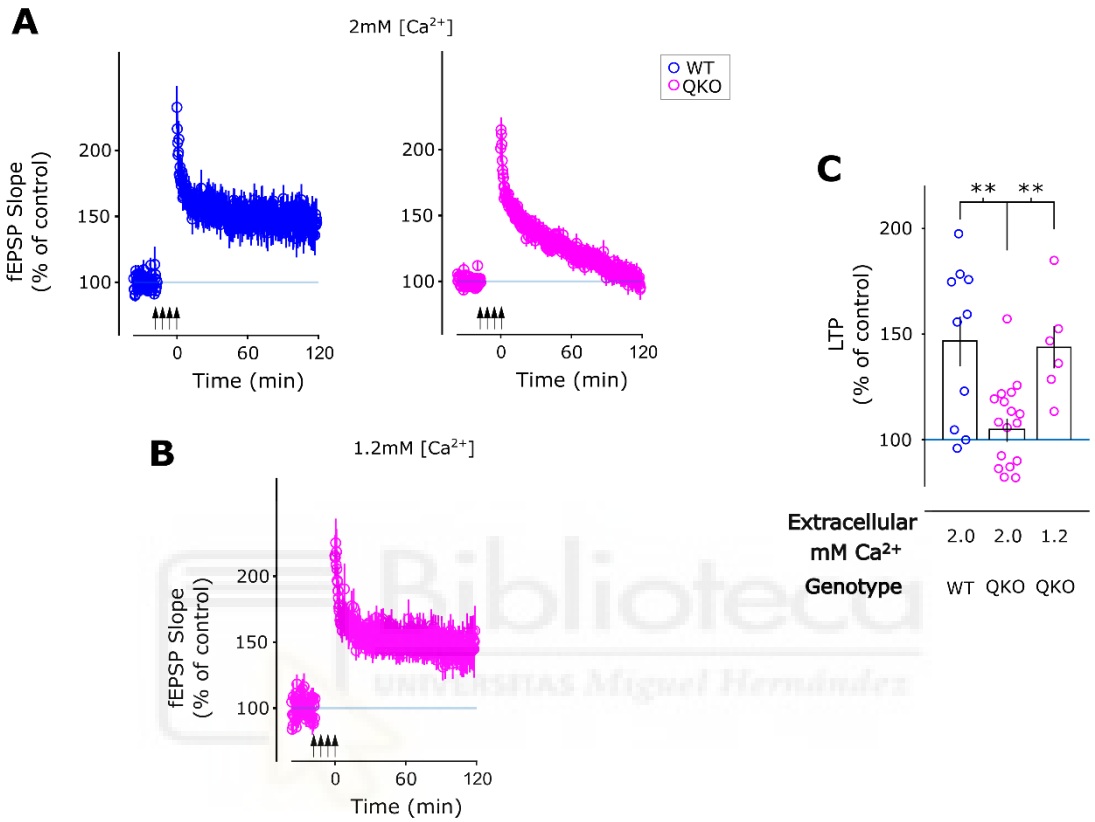


Figure 6 L-LTP is also occluded in QKO but rescued by low Ca^{2+} (A) 4 trains of 1s at 100 Hz separated by 5 minutes (arrows) elicit a potentiation stable in WT but that is lost with time in QKO. (B) The potentiation is kept in QKO when Ca^{2+} is lowered to 1.2 mM. (C) After LTP induction, WT shows bigger potentiation at 110-120 min (146 ± 12% n = 10, mice = 9) than QKO (104 ± 6% n = 18, mice = 14) (** signifies p<0.01, rank sum). In 1.2 mM QKO shows stable potentiation (143 ± 10% n = 6, mice = 3) compared with 2 mM results (** signifies p<0.01, rank sum).

Subsequent experiments showed that L-LTP induced in 1.2 mM Ca^{2+} was similar at WT and QKO synapses, and the amount of potentiation was close to the maximum we could attain with extra stimulations protocols like those used in Figure 3 (Figure 7). Taken together, these results indicate that the longest-lasting components of LTP are blocked at QKO Schaffer collateral synapses when extracellular Ca^{2+} is 2 mM, but not when Ca^{2+} is 1.2 mM.

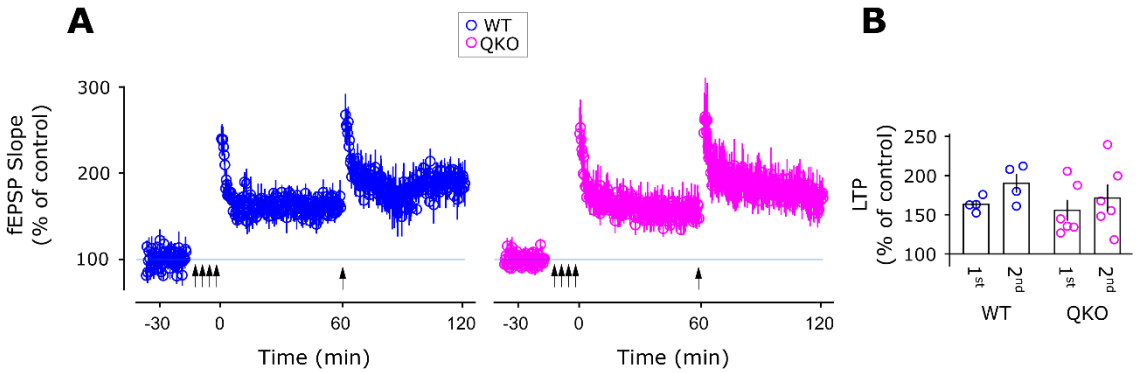


Figure 7 L-LTP protocol closes potentiation to the maximum. (A)Comparison of LTP at WT (n = 4, mice = 3) and QKO synapses in 1.2 mM Ca^{2+} (n = 6, mice = 5). LTP was first induced with 4 trains of 100 Hz stimulation as in Figure 6 and then, after 60 min, with the standard 2 train protocol as in Figure 3. (B) LTP was quantified at 51 - 60 after induction respect to the mean baseline (WT 163 ± 6%; QKO 156 ± 15%) after the 1st induction protocol and for minutes 111 – 120 for the 2nd (WT 190 ± 14%; QKO 171 ± 19%).

1.4 Expression is occluded at QKO synapses, but induction is intact

With the previously observed results, we considered that p_v may play a role in LTP stability. However, it may be affecting different steps which together shape the potentiation. To look deeper into the possible processes in which p_v is involved, we grouped them into two categories according to their part in LTP: induction and expression.

Unlike STP, which lasts no more than a few minutes, LTP duration is sufficient to allow modifications in p_v at different points along the entire event. By changing the p_v at distinct times during the experiment, we aimed to discern where the target lies. If the LTP induction is performed with a Ca^{2+} concentration which occludes potentiation, but LTP expression is recorded at low p_v conditions, we might be able to discern where occlusion is occurring.

We decided to use QKO due to the results observed in LTP previously. Conditions of 1.2 mM Ca^{2+} from previous experiment where LTP was stable in QKO were repeated. After a stable baseline, ACSF was substituted for another one with 2

mM Ca^{2+}. A new baseline was then obtained once ACSF was completely changed with subsequent LTP induction protocol as described in the previous experiment (Figure 6). After induction, ACSF was changed back to the previous one with a 1.2 mM Ca^{2+} concentration. Then, responses were recorded for at least 90 minutes (Figure 8.A). The first baseline obtained at 1.2 mM Ca^{2+} was used as reference for the LTP expression recorded at the same Ca^{2+} concentration after induction at 2 mM Ca^{2+}. Here, QKO synapses showed a potentiation during the recording at 1.2 mM Ca^{2+} compared with the initial baseline.

A control experiment was performed in parallel. In the same bath chamber, another slice stimulation was recorded at the same time. Instead of LTP induction, after ACSF changing to 2 mM Ca^{2+}, no stimulation protocols were applied. In this case, no LTP was observed after returning to the initial 1.2 mM Ca^{2+} solution (Figure 8.C). The fEPSP responses returned to their previous level before changing to 2 mM Ca^{2+} without any kind of potentiation. The purpose of this control was to eliminate any possible effects on synaptic responses caused by the change in Ca^{2+} concentration.

To rule out the possibility of modifications in the fEPSP caused by the low Ca^{2+} ACSF before LTP induction, the experiment was replicated but keeping the 2 mM Ca^{2+} ACSF after tetanus stimulation (Figure 8.B). This time, QKO did not maintain potentiation after 90 minutes with respect to the new 2 mM Ca^{2+} baseline, consistent with the previous results (Figure 6.B).

These findings suggest that the induction of LTP remains unimpeded at QKO synapses under 2 mM Ca^{2+} conditions, thus implying that the deficit observed at 2mM Ca^{2+} must manifest at the level of expression.

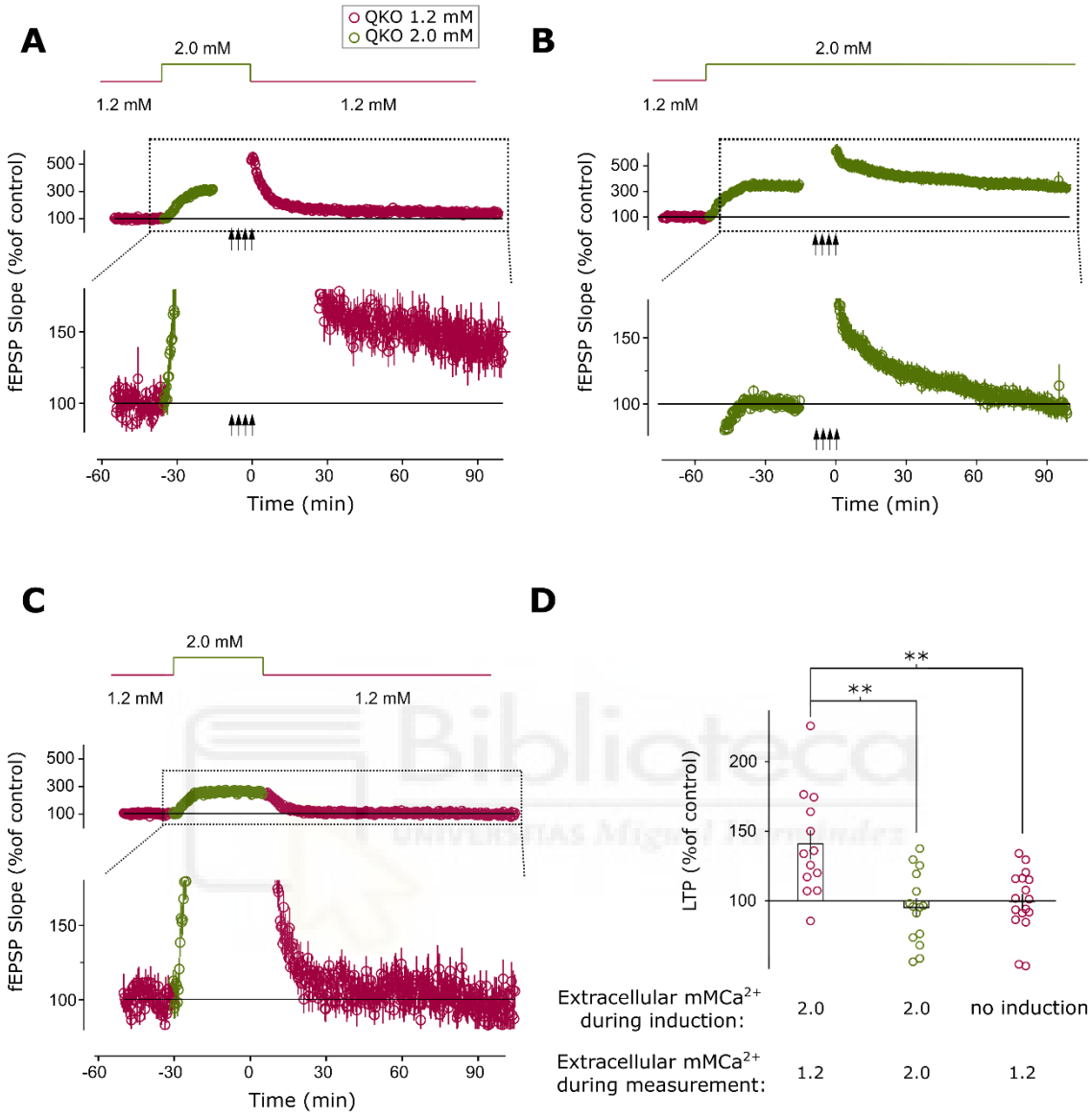


Figure 8 Expression but not induction of LTP is occluded in QKO. (A) QKO synapses show stable potentiation in 1.2 mM extracellular Ca^{2+} respect to the baseline at the same concentration when the LTP induction is at 2 mM extracellular Ca^{2+} (n = 13, mice = 13), but not (B) when is kept 2 mM after induction (n = 15, mice = 15). (C) Generate a baseline 1.2 mM Ca^{2+} and increase it to 2 mM does not generate any kind of potentiation (n = 17, mice = 10). (D) The induction of LTP in 2 mM Ca^{2+} generates a potentiation which can be observed in 1.2 mM Ca^{2+} (140 ± 10%) but not in 2 mM (95 ± 6%) (** signifies $p<0.01$, rank sum), like just the change of Ca^{2+} alone (99 ± 5%) (** signifies $p<0.01$, rank sum).

1.5 Impact of Ca^{2+} reduction

The reduction of extracellular Ca^{2+} from 2 to 1.2 mM resulted in a decrease in baseline synaptic strength by a factor of 2.6 ± 0.1 at QKO synapses (n = 15; see Figure 8.C), likely attributable to a proportional decrease in p_v. We tested whether this reduction in Ca^{2+} did not affect the postsynaptic response by measuring spontaneous miniature synaptic currents, commonly referred to as "minis," in CA1 principal neurons (Figure 9).

The measurements obtained by whole-cell recordings of CA1 neurons showed no difference in the mini cumulative probabilities at different Ca^{2+} concentration. This confirms that the diminution in synaptic strength following Ca^{2+} reduction primarily occurs via a presynaptic mechanism. These results lend support to the hypothesis that deficits in LTP at QKO synapses stem from an elevation in baseline p_v rather than deficiencies in underlying mechanisms.

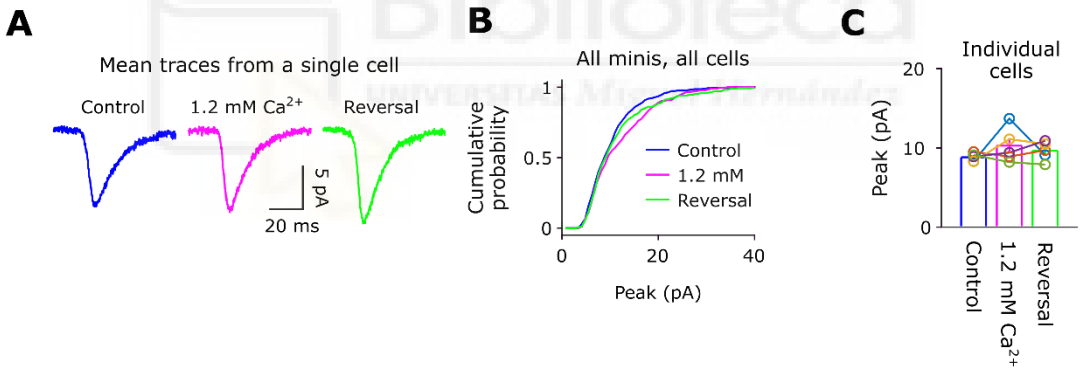


Figure 9 Minis size and probability in QKO. No change in size of minis when lowering Ca^{2+} to 1.2 mM at QKO synapses. Minis were recorded in whole cell voltage clamp during 5 min intervals first when extracellular Ca^{2+} was 2 mM, then after changing to 1.2 mM, and finally a third time after returning to 2 mM. Experiments were excluded if the access resistance changed more than 10 % (n = 5, mice = 2). (A) Mean electrophysiological traces for a single neuron. (B) Cumulative probability versus peak amplitude for all minis from all neurons. (C) Mean peak size of all minis from the individual neurons (Control 8.9 ± 0.2, 1.2 mM Ca^{2+} 10.3 ± 1.0, Reversal 9.6 ± 0.5 pA).

1.6 Occlusion of expression at WT Synapses

If our assumptions are correct, LTP is elevating the p_v, but it cannot be observed in QKO synapses at normal conditions because their native increased p_v is occluding this new enhancement. So, elevating baseline synaptic strength at WT synapses by increasing extracellular Ca^{2+} would similarly impede the expression but not the induction of LTP.

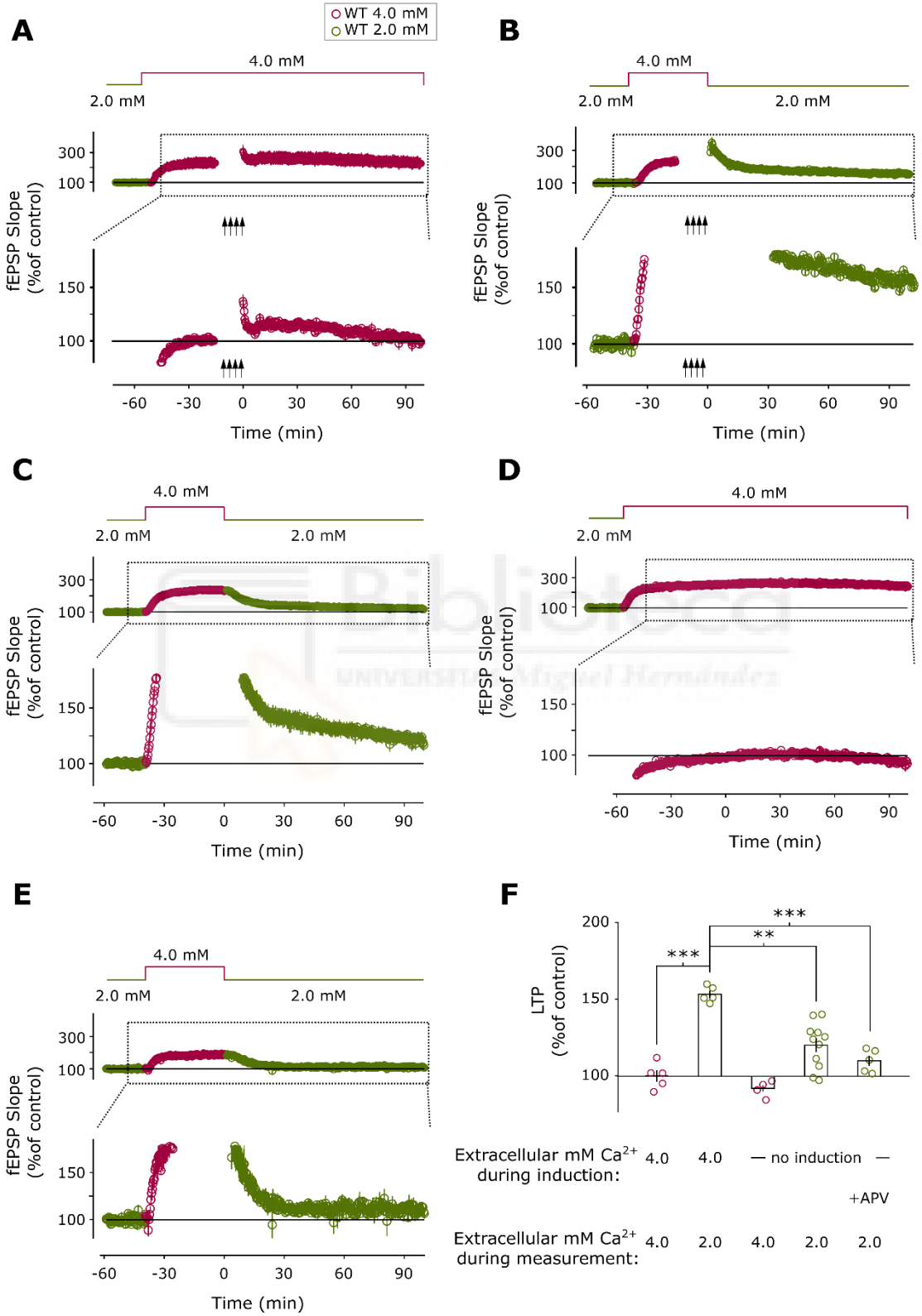
Instead of starting from low Ca^{2+} and moving to a normal concentration, this time we moved from normal to high levels of Ca^{2+}. We wanted to reproduce a normal p_v at the beginning and an enhanced one during the induction, as in the QKO synapse experiment (Figure 8). The Ca^{2+}/Mg^{2+} concentration used was 2/2 mM for normal p_v and 4/0.5 for the enhanced condition.

We were first looking to replicate in WT the phenotype in QKO at 2 mM Ca^{2+}, no LTP after some time. Therefore, we started with a baseline at 2 mM Ca^{2+} and moved to 4 mM. Once responses were stable, we induced LTP and measured responses for at least 90 minutes (Figure 10.A). As expected, after 90 minutes fEPSPs were equal to 4 mM Ca^{2+} baseline levels.

As a parallel control, another slice in the same bath chamber was recorded without induction (Figure 10.D). The fEPSPs remained stable during the whole time the other experiment lasted, ruling out excitotoxicity problems.

To determine, as before, whether the problem was in the induction or the expression, after tetanus stimulation, ACSF was changed back to the initial one with 2 mM Ca^{2+} (Figure 10.B). A stable LTP appeared compared to the initial baseline, with similar potentiation levels to those observed in QKO at low p_v conditions (see Figure 6.B). So, LTP induction seems to be intact in enhanced p_v conditions caused by high Ca^{2+}. On the other hand, LTP expression cannot be observed if the p_v is already elevated.

In this occasion, the parallel control with returning to initial ACSF but no 100 Hz tetanus stimulation, as in the QKO experiment, showed some residual potentiation with respect to the baseline (Figure 10.C). Despite this, the potentiation observed between induction and no induction was significantly different in the first one. We added another extra control like the original but adding APV to the bath. In this case the presence of the NMDAR blocker halved the residual potentiation, so now fEPSPs are less than 10% bigger after high Ca^{2+} conditions (Figure 10.E).


A
WT 4.0 mM
WT 2.0 mM
4.0 mM
2.0 mM
fEPSP Slope (%of control)
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B
4.0 mM
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C
4.0 mM
2.0 mM
2.0 mM
D
4.0 mM
2.0 mM
E
4.0 mM
2.0 mM
2.0 mM
F
LTP (%of control)
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Extracellular mM Ca2+ during induction: 4.0 4.0 — no induction — +APV
Extracellular mM Ca2+ during measurement: 4.0 2.0 4.0 2.0 2.0

Figure 10 Expression but not induction of LTP is occluded in WT at high Ca^{2+}. (A) In WT mice there is no stable LTP at 4/0.5 mM extracellular Ca^{2+}/Mg^{2+} (n = 5, mice = 5), (B) but if Ca^{2+}/Mg^{2+} is lowered back to 2/2 mM after induction potentiation is stable (n = 5, mice = 5). (C) The Ca^{2+} increase generates a residual potentiation respect to the baseline (n = 11, mice = 9). (D) High Ca^{2+} seems to not be harmful for the stability of the fEPSPs (n = 5, mice = 5). (E) Residual potentiation is reduced in the presence of APV (n = 5, mice = 4). (F) Induction of LTP at 4.0 mM Ca^{2+} generates a stable potentiation that cannot be observed at the same concentration (99 ± 3%) but yes at 2 mM (153 ± 2%) (*** signifies p<0.001, rank sum). LTP at 2 mM is higher than residual potentiation independently of the presence of APV (109 ± 3%) (*** signifies p<0.001, rank sum) or not (119 ± 4%) (** signifies p<0.01, rank sum).

We thought that spontaneous opening of NMDARs in addition to evoked at low frequency stimulation, which in normal conditions is harmless, here may generate some LTP. Under normal conditions, these odd openings do not allow the entrance of too much Ca^{2+}. But due to the high concentration of Ca^{2+} achieved during the experiment and the lower Mg^{2+} concentration, these apertures provoke the entrance of bigger amounts of Ca^{2+}. This might be starting LTP processes at a minor scale.

These results coincide with those showed previously, confirming the role of p_v in LTP. Furthermore, the ability for blocking LTP in WT independently of Syp family makes unlikely that they have a role underlying LTP mechanisms apart from its influence in p_v under our research conditions.

2. LTD is also affected by p_v

LTD presents the same controversies as LTP: which are the roles of pre- and postsynaptic sides of synapse and which are the underlying mechanisms. Our goal was to investigate whether p_v affects also LTD and whether LTD previous to LTP can rescue the potentiation in QKO.

A blind experiment for WT and QKO mice hippocampus slices was designed to answer both questions simultaneously. A baseline of at least 30 minutes at 0.05 Hz, with a Ca^{2+}/Mg^{2+} concentration of 2/2 mM, was obtained using extracellular recordings, similar to previous LTP experiments. An LTD protocol of 900 pulses at 1 Hz (Dudek & Bear, 1992) was applied to both WT and QKO. 30 minutes later, LTP was induced by a tetanus protocol consisting of 5 trains of 200 ms at 100 Hz separated by 10 s. The posterior EPSPs were recorded for at least 60 more minutes (Figure 11.A).

At the end of the experiment, LTP levels compared with the initial baseline were similar to those observed in previous experiments (Figure 11.B). In QKO, potentiation

returned to baseline levels after 60 minutes, while it remained stable in WT after that time. Apparently, previous induction of LTD does not rescue the QKO phenotype for LTP at normal Ca^{2+} levels, at least not LTP above the original baseline value.

Interestingly, LTD appeared to be more pronounced in QKO than in WT mice (Figure 11.C). 30 minutes after LTD induction, synaptic depression was almost lost in WT, whereas QKO still exhibited a decrease in the slope of fEPSPs. This could be interpreted as Syp family having a role in LTD or as the enhanced p_v affecting depression.

Despite the lack of rescue of LTP by LTD, we decided to measure relative enhancement according to the depressed responses. Using the LTD levels as baseline, measured 10 minutes before the LTP protocol, yielded significantly different results regarding potentiation (Figure 11.D). In this case, QKO exhibited LTP compared to the new baseline, aligning with the previous experiment where p_v was reduced. With LTD levels as baseline, WT LTP was only slightly higher and not statistically different from that observed in QKO.

The likelihood of Syp family proteins by itself affecting LTD now seems unlikely. If QKO synaptic depression were influenced by a phenomenon unrelated to p_v, LTP re-potentiation using LTD as baseline would have shown unstable potentiation, as in previous experiments. For this reason, p_v may also be influenced by LTD. We have observed how a reduced p_v enables QKO to express LTP, as seen in this experiment. Hence, we may conclude that LTD rescues relative LTP at QKO synapses by modifying the p_v.

Moreover, the difference in LTD levels might open the door to new theories. The reduced depression in WT compared to QKO suggests a potential new framework. Until now, our focus has been on the upper limitations of p_v and its inability to be over-enhanced. Nevertheless, QKO mice showing a higher LTD might indicate that there is not only an increment occlusion for p_v. A logarithmic dynamic for p_v changes could explain why WT showed less depression but more LTP. Their initial p_v allow them to get potentiated but not too much depressed. In contrast, QKO starts with an enhanced p_v, preventing them from increase the synaptic response too much but allowing for a greater decrease than in WT. As mentioned earlier, this presents a new framework that requires further study.

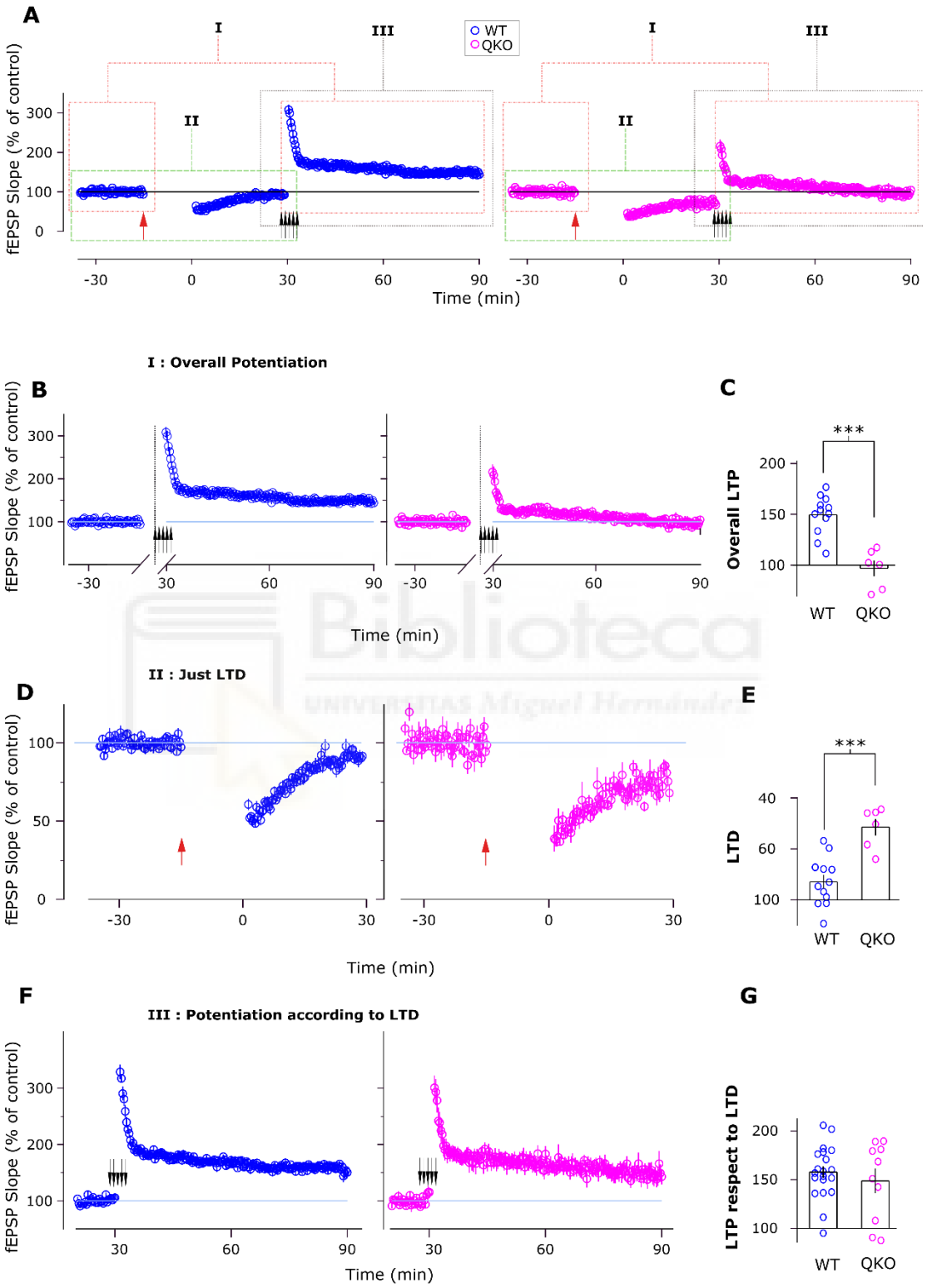

Figure 11 LTP is relatively rescued by LTD in QKO. (A) After a baseline of at least 20 min LTD is inducted (900 pulses at 1 Hz, red arrow) for 30 min later induce LTP (5 trains of 200 ms at 100 Hz separated by 10 s, black arrows). (B-C) Potentiation after 60 min compared with the baseline is stable in WT (149 ± 5%, n = 12, mice = 6) but disappears in QKO (96 ± 7%, n = 6, mice = 6) (*** signifies p<0.001, rank sum). (D-E) LTD induction causes more depression in QKO (71 ± 3%, n = 6, mice = 6) than in WT (92 ± 2%, n = 12, mice = 6) (*** signifies p<0.001, rank sum). (F-G) Comparing potentiation with responses 30 minutes after LTD instead of initial baseline, the relative potentiation after LTP induction turns stable in QKO (148 ± 12%, n =10, mice = 5) and bigger in WT (157 ± 6%, n = 20, mice = 8).

3. p_v enhancement occlusion is also plasticity independent

Although we could ensure that p_v is modified by extracellular Ca^{2+}, the occlusion observed during LTP expression might not be caused by the own p_v. In case p_v increment by Ca^{2+} were similar between QKO and WT, it would open the door to more possible candidates for LTP occlusion. A mechanism occluded by the enhanced p_v, but not an occlusion in the own p_v increase could be the responsible for the phenotype.

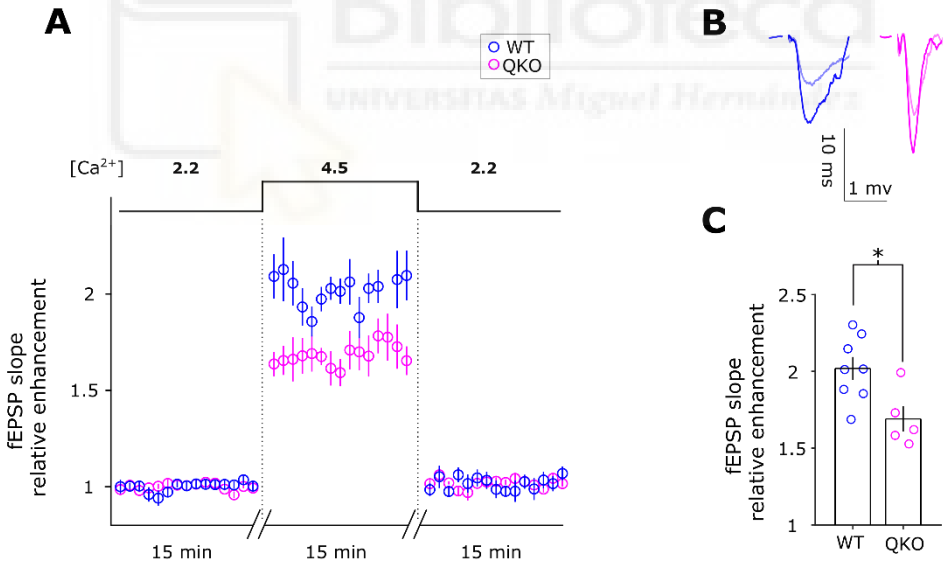

Figure 12 Synaptic potentiation by high Ca^{2+} is limited in QKO. (A) After a baseline of at least 15 minutes extracellular Ca^{2+} is elevated from 2.2 mM to 4.5 mM (representation skipped) until stabilization of the response for at least another 15 minutes and then ACSF is changed back 2.2 Ca^{2+}. Experiments where responses do not come back to baseline levels after were ruled out. (B) Representative examples of how fEPSPs grow. (C) The increasing in the slope respect to the baseline produced by the higher Ca^{2+} concentration is bigger in WT (2.01 ± 0.08, n = 8, mice = 5) than in QKO (1.59 ± 0.09, n = 5, mice = 2) (* signifies p<0.05, rank sum).

We tested the occlusion in the p_v enhancement repeating the previous experiment where we measured synaptic responses at different Ca^{2+} concentration. But this time we would compare QKO and WT blindly at similar conditions. Baseline was stablished at 2.2/2.6 mM Ca^{2+}/Mg^{2+} and ACSF would be changed to 4.5/0.5 mM Ca^{2+}/Mg^{2+}. Only those experiments where synaptic fEPSP slopes were like the baseline after returning to initial ACSF were accepted. APV was added to the bath in order to avoid residual potentiation.

Comparing the mean responses during 4.5 mM Ca^{2+} with the baseline obtained in 2.2 mM Ca^{2+}, we found that slope size was doubled in WT. In contrast, QKO fEPSPs were only 59% bigger (Figure12.C).

Thanks to this control we confirmed that p_v growing has a limit independent of plasticity, and that is reached earlier in QKO synapses due to their native enhanced state. Furthermore, it supports the original explanation for the previous results: occlusion of LTP expression is caused by a limit in the p_v enhancement.

4. STP is occluded by enhanced p_v

All the previous results seem to confirm that p_v is modified during Long-Term Plasticity, especially in LTP. However, this enhancement is occluded when p_v is already increased, like in QKO or high Ca^{2+} extracellular conditions. This phenotype is similar to the one showed in Raja et al., 2019, where facilitation was occluded in QKO. The p_v as common element for the occlusion of two events apparently un related lead us to think that maybe this could be shared among more synaptic plasticity cases.

For this reason, we decided to check how Syp family absence, and therefore enhanced p_v, affect to different kinds of plasticity. This would reveal if really p_v is ubiquitously modified during plasticity or these are just single cases.

4.1 PTP reduced at calyx of Held

We began our testing at the calyx of Held, in line with our previous studies on this synapse, where facilitation was occluded in QKO synapses (Raja et al., 2019). As in facilitation, Syp family deletion might affect PTP because its potentiation arises from a temporarily enhanced p_v.

To induce PTP, a 100 Hz stimulation train for 4 s was applied, and posterior responses at 0.1 Hz during at least 3 minutes were compared with a previous baseline of 1 minute. A 20 s resting time after the induction was selected based on the time necessary restoration of the RRP in QKO mice (Raja et al., 2019). This prevents any possible decrease in the size of EPSCs due to depletion, which would mask potentiation.

As expected, WT synapses exhibited increased EPSCs compared to the baseline levels while QKOs displayed a smaller enhancement, though not entirely absent (Figure 13.A). Again, elevated p_v in WT animals by increasing extracellular Ca^{2+} from 2 mM to 4 mM to mimic the QKO phenotype reduced potentiation (Figure 13.B). The KYN concentration was doubled from 1 to 2 mM to prevent AMPARs desensitization.

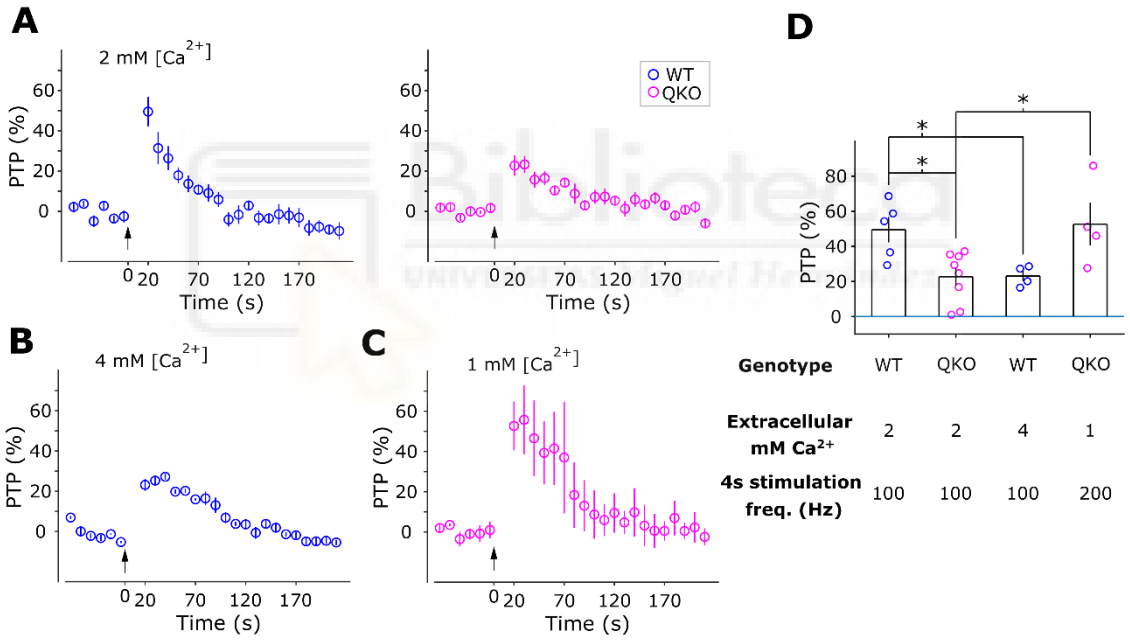

Figure 13 PTP is affected by p_v in calyx of Held. (A) Potentiation at 2 mM Ca^{2+} in WT (n = 5, mice = 4) and QKO (n = 8, mice = 8) after a 4 s 100 Hz train. (B) Potentiation is reduced in WT when Ca^{2+} is raised to 4 mM Ca^{2+} (n = 4, mice = 3) and recovered (C) in QKO when Ca^{2+} is lowered to 1 mM and stimulation frequency is doubled (n = 4, mice = 4). (D) Statistic analysis confirm how potentiation is different between WT (149 ± 7%) and QKO (122 ± 5%) under same conditions (* signifies p<0.05, rank sum). Potentiation in WT at 4 mM Ca^{2+} (123 ± 12%) is significantly smaller than in normal conditions (* signifies p<0.05, rank sum), and higher in QKO in 1 mM Ca^{2+} (152 ± 12%) respect to 2 mM (* signifies p<0.05, rank sum).

On the other hand, halving Ca^{2+} concentration to 1 mM in QKO resulted in a similar amount of enhancement in the size of EPSCs compared to the baseline, as in WT synapses under 2 mM Ca^{2+} conditions (Figure 13.C). Additionally, tetanus stimulation was increased to 200 Hz, compensating for the reduced Ca^{2+} entry into the presynaptic terminal during each action potential. This increased frequency ensured the accumulation of residual Ca^{2+}.

The results obtained were consistent with what our lab previously shown about facilitation in QKO, so as with those described about LTP. Hence, WT PTP levels are bigger than QKO under standard conditions, but the phenotype can be altered by changing p_v thanks to extracellular Ca^{2+} (Figure 13.D).

Although LTP in Schaffer Collaterals and PTP in calyx of Held are different in synaptic origin, both plasticity events present changes in p_v during its expression. This reinforces the idea of p_v alterations as shared mechanism for synaptic plasticity expression.

4.2 PTP reduced at hippocampus

Once we confirmed that PTP expression is occluded by enhanced p_v, we moved to another synapse to check the reproducibility of the phenomenon. The hippocampus was chosen due to previous experiments of LTP

This time we used a 50 Hz 1 s train induction because Schaffer collateral fibers do not support stimulations as high as anteroventral cochlear nucleus axons. A previous recording of 2 min at 0.05 Hz stimulation frequency was used as baseline for the posterior measurement of PTP. 20 s after tetanus stimulation, PTP was measured for at least 4 minutes at 0.05 Hz. Experiments were done at 2.6 mM extracellular Ca^{2+} and 1.3 mM Mg^{2+}, with APV addition to the ACSF.

As expected, first measurement after tetanus stimulation showed a bigger PTP in WT than in QKO synapses (Figure 14.A). However, after repeating the experiment in 1.2 mM Ca^{2+} and 2 Mg^{2+}, PTP was not recovered in QKO, while WT potentiation was still higher (Figure 14.B).

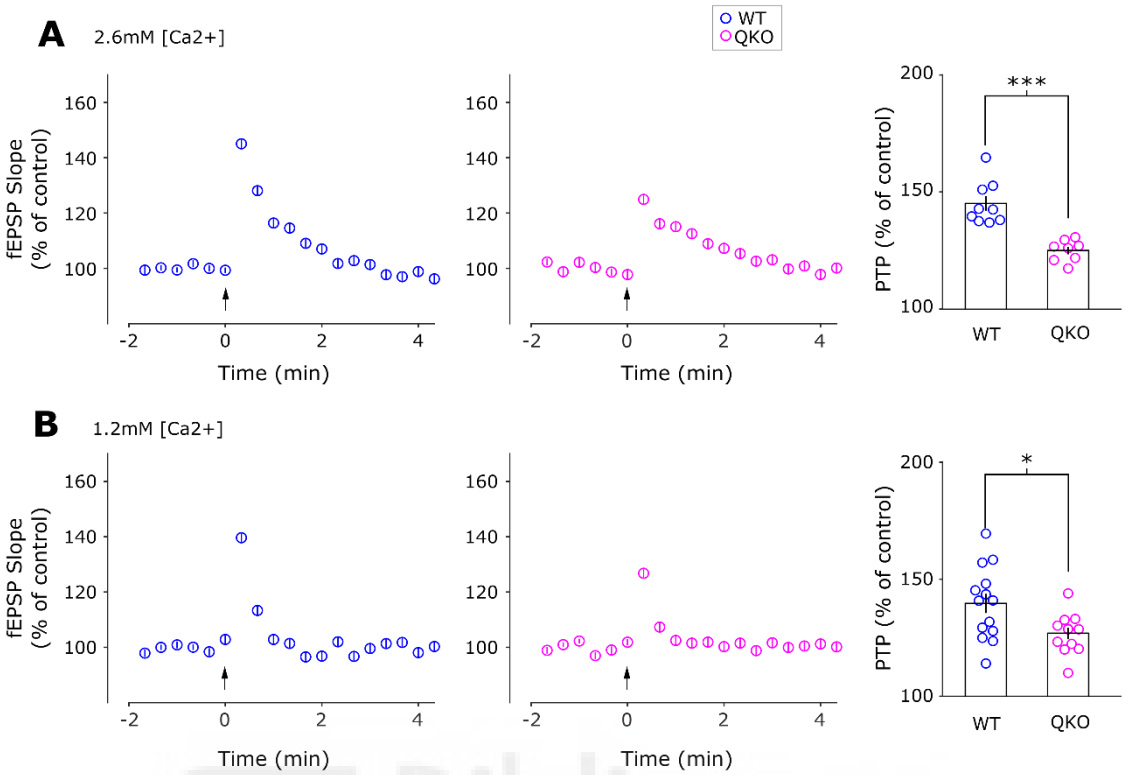

Figure 14 PTP is also reduced in QKO in hippocampus. (A) At 2.6 extracellular mM Ca^{2+}, potentiation evoked by a tetanic stimulation of 50 Hz during 1 s (arrow) causes a bigger effect in WT animals (145 ± 3%, n = 9, mice = 2) respect to the QKO (124 ± 2%, n = 8, mice = 2) (*** signifies p<0.0001, rank sum). (B) With a lower Ca^{2+} concentration of 1.2 mM, difference is slightly smaller (WT 139 ± 4%, n = 14, mice = 3; QKO 126 ± 2%, n = 12, mice = 2) but persists (* signifies p<0.05, rank sum).

Despite the apparently negative results, we maintain our hypothesis. The lack of potentiation in QKO under low p_v conditions might be explained because of the Ca^{2+} entrance to the presynaptic terminal. A low extracellular Ca^{2+} concentration together with a relatively low tetanus stimulation provokes less Ca^{2+} accumulation in the presynaptic terminal. This would make that potentiation decays faster due to the low Ca^{2+} concentration. So, PTP enhancement was present, but at the measurement point, potentiation decay has already started.

Nonetheless, a reduction in STP potentiation in QKO compared with WT is consistent with our previous results.

Discussion

This thesis had three distinct objectives. First, inspired by the research by Janz and Südhof, we aimed to investigate if Syp family has a role on LTP. Second, we sought to determine a possible role for p_v in LTP. Finally, we wanted to determine if the occlusion observed in our previous research on Raja et al. in QKO synapses was a common feature in different plasticity events.

The first two objectives were pursued simultaneously because of the inherent enhanced p_v of the QKO mice synapses, used both as tool for the LTP study and as a research focus. The ubiquitous occlusion of the p_v enhancement through plasticity was demonstrated, serving as control for the initial theories.

The role of p_v showed during this thesis contributes to the extensive list of evidences confirming factors affected by LTP. However, its significance might extend beyond its immediate implications. The debate about how LTP is expressed is far from being resolved, but this addition includes some pivotal points. While presynaptic modifications during LTP in mossy fibers synapses are widely accepted, they are not so commonly reported for Schafer collaterals.

Most of the research on these synapses focus on the postsynaptic changes, like AMPAR externalization or de novo protein synthesis in the dendritic spine. Indeed, many of them show no presynaptic modifications. But here is one more evidence of presynaptic changes in Schaffer collaterals during LTP.

The results from this thesis contribute to an idea that goes further than the debate of pre- versus postsynaptic modifications. There is no doubt about the relevance of postsynaptic changes during LTP, we are just adding pieces to the puzzle that is its mechanism. The whole complexity of LTP is not close of being understood, although the data shown here help to connect some evidences that were already on the table.

Future experiments combining the occlusion of LTP at different points of the postsynaptic mechanism responsible for expression, or another presynaptic modification different from those explained in this thesis, might contribute to gaining new insights. The moment where mechanisms underlying LTP change from postsynaptic to presynaptic could be unveiled, so as persistence of each one of those mechanisms.

In addition to the results on LTP, the findings obtained in other synapses and events presented during the thesis might help to a better understanding about some general plasticity concepts.

Synaptophysin family has an indirect role in plasticity

Synaptophysin is a family of proteins abundant in the presynaptic terminals of the CNS. Its functions are not completely clear, and how its role regulating p_v relates with the plasticity mechanisms is even less understood.

The initial experiments of this research were comparing LTP at QKO and WT Schaffer collateral synapses. Potentiation decreased to baseline levels at QKO synapses after an hour (Figure 3), but it was rescued when experiments were performed in low extracellular Ca^{2+} conditions, which lowers p_v to WT levels (Figure 5). This result differs from that obtained by Janz and Sudhof, where LTP was simply reduced in the double KO synapses compared to WT at all times after induction. Notably, however, the difference might be because QKO synapses are missing all four members of the Syp family, or because extracellular Mg^{2+} was 1 mM in the Janz study – compared to 2 mM in ours – which would increase baseline p_v.

Our initial hypothesis was that the absence of the entire Syp family was reducing the lasting LTP, suggesting that a more persistent plasticity modification might be needed. However, the same deficit occurred with a stronger protocol, inducting L-LTP this time: potentiation was occluded in normal Ca^{2+} at QKO synapses and recovered when extracellular Ca^{2+} was reduced (Figure 6). In fact, in Figure 7, we observed how repeated induction protocols did not rescue potentiation in QKO synapses under 1.2 mM Ca^{2+} conditions indicating that the level attained was close to the maximum. Lowering Ca^{2+} reduces p_v, countering the effect of Syp family absence, clearly indicating that Syp family members do not play an essential role in LTP. The enhanced p_v already present in the QKO synapses might be the responsible.

However, LTP is a process with many steps involved, so it was not clear where the Syp family might be acting. By recording our experiments in low Ca^{2+} conditions, where we had previously observed stable LTP in QKO synapses, but inducing it in normal Ca^{2+} levels, where potentiation was occluded, we expected to discern in which point of the whole event the occlusion was happening. We observed that regardless of the Ca^{2+} levels during the induction, LTP expression was only stable in QKO synapses under low Ca^{2+} conditions (Figure 8), and therefore a state of low p_v. This located expression over induction as responsible for the phenotype observed in the initial experiments. Even if this result confirmed a role for p_v, it did not rule out completely the possibility that Syp family was responsible for this phenotype instead of the enhanced p_v caused by its absence.

To explore more deeply, we decided to repeat the previous experiment at WT synapses. The already enhanced p_v from QKO synapses was mimicked by enhancing extracellular Ca^{2+} during induction of the LTP protocol, and the results recapitulated those observed in QKO synapses. Independently of the conditions during induction, LTP can only be expressed when p_v is not already enhanced (Figure 10). This experiment confirmed that the observed phenotype was not directly caused by the absence of the Syp family, but rather by the enhanced p_v it provokes.

A reevaluation of the possible roles for Syp family is therefore required. As mentioned in our previous studies and confirmed during this thesis, it regulates p_v. This phenomenon, probably caused by interacting with VAMP2, may influence in the expression of plasticity modifications.

Many studies suggest that Short-Term Plasticity act as a filter or modulator for posterior Long-Term Plasticity modifications. Short-Term Plasticity might contribute to maximize information transfer or to the tuning of the area-specific activity for Long-Term Plasticity and spatio-temporal processing (Deperrois & Graupner, 2020; Dudman et al., 2007; Fortune & Rose, 2001; Rotman et al., 2011).

Indeed, we observed in Figure 4 how the initial part of LTP was reduced. This might mean that Syp is part of a mechanism responsible of controlling plasticity. If changes in p_v are one of the targets for expression of synaptic potentiation, regulation of p_v becomes necessary.

The existence of mechanisms occluding the over potentiation of synapses that have already undergone LTP would help to avoid overlapping of potentiation in distinct neuron populations, preventing unrelated memories from being created. Although the number of synaptic connections in the brain is large, it is not infinite, so it is necessary to regulate which ones are potentiated.

Syp family regulation of p_v affects the expression of plasticity and may regulate indirectly the formation of new memories. Unfortunately, some behavioral tests were performed without any differences observed between QKO and WT. Probably this is due to the physiological Ca^{2+} levels in the brain, which are lower than those where LTP is observed in QKO.

p_v is modified during Long Term Plasticity

It is known that during LTP, p_v is modified in mossy fiber synapses. On the other hand, the responsibility for LTP expression in Schaffer collaterals was attributed during years to postsynaptic modifications. There has already been some evidence of presynaptic modifications in Schaffer collaterals, but LTP expression had not been occluded completely yet due to only presynaptic mechanisms.

All the results exposed during the previous section point to p_v as responsible for the expression of the LTP. We have been able to observe, occlude, mimic and block how p_v regulates LTP expression. There is LTP in WT synapses with no alterations in p_v (Figure 3). This LTP expression is occluded by the enhanced p_v in QKO synapses. We could mimic the occlusion in WT synapses by enhancing extracellular Ca^{2+} (Figure 10). Indeed, we have even been able to prevent the occlusion observed in QKO, expressing LTP without changing extracellular Ca^{2+}.

In experiments shown in Figure 11, a depression of the synaptic response before the induction of LTP allows the relative potentiation of those synapses. In QKO synapses the expression of LTP with respect to the initial baseline was abolished after 90 minutes, while it remained in WT synapses. But when we used as baseline the depressed responses after LTD induction, both QKO and WT synapses showed stable potentiation.

As for LTP, LTD in Schaffer collaterals is usually explained by postsynaptic mechanisms. But again, there are some studies showing how LTD affects p_v in these synapses (Andrade-Talavera et al., 2016; Baltaci et al., 2019; Bayazitov et al., 2007; P. E. Castillo, 2012). So, these studies are supported by results in Figure 11 and vice versa. A reduced p_v caused by the LTD protocol would turn down p_v enhancement in QKO synapses to WT levels, allowing therefore the expression of LTP.

The postsynaptic mechanisms for LTP and LTD are similar but working in opposite directions: the trafficking of AMPARs. Therefore, the idea of p_v affecting both LTP and LTD seems quite reliable too. Future experiments for confirming the role of p_v in LTD will be needed. An experiment inspired on those from figures 8 and 10 might be useful for discerning if under same p_v conditions, WT and QKO synapses get equally depressed.

The possibility of an unidentified mechanism affected by the changes in Ca^{2+} concentration as responsible for all the phenotypes observed was considered. Nevertheless, other mechanisms besides alterations of p_v as responsible for the occlusion were discarded thanks to the experiments in figure 9 and 12. First, no differences in the mini cumulative probabilities were observed during the changes in extracellular Ca^{2+} concentration (Figure 9). This implies no alterations in postsynaptic properties, and therefore only changes via presynaptic machinery. On the other hand, the amount of potentiation was lower in QKO synapses compared to WT when we increased extracellular Ca^{2+} in figure 12. A similar enhancement in the synaptic responses of both genotypes would have meant that p_v is not affected by Ca^{2+} or that it has no limit in its enhancement. Instead, the fact that synaptic responses were relatively more increased in WT synapses means the opposite: there is a limit in the p_v enhancement, which is the factor affected by the Ca^{2+} increase.

How p_v is modified during LTP cannot be determined yet. Many theories exposed in other studies could be the starting point for new research. Retrograde messengers or trans synaptic proteins activated thanks to the Ca^{2+} influx through NMDARs might explain how presynaptic changes are initiated besides the postsynaptic modifications.

p_v enhancement is a shared plasticity mechanism

Short-Term Plasticity is usually explained by changes in presynaptic terminals. Most of the time, these changes affect the p_v. Previous studies had already related facilitation and LTP (Kullmann, 2012; Yasui et al., 2005), suggesting a possible link between Short- and Long-Term Plasticity. Supporting those theories, here we show the occlusion of potentiation in different synapses and plasticity events thanks to a shared mechanism.

Regarding the idea of testing if p_v enhancement of QKO was affecting plasticity from a general perspective, we tested Short-Term Plasticity. Our previous results already showed how facilitation was occluded in QKO synapses and could be rescued by lowering Ca^{2+}. Although it supports the idea of p_v affecting plasticity transversally, testing more events would reinforce this idea.

Results in Figure 13, where PTP in calyx of Held is occluded in QKO but not in WT, align with our previous results. Again, potentiation can be occluded by altering p_v and rescued thanks to lowering Ca^{2+} in QKO synapses. This not only reinforces the idea of p_v affecting the expression of synaptic potentiation in every plasticity event, also proves how it is something common among different synapses. Unfortunately, these

results were not as clear on Schaffer collaterals (Figure 14), but this might be due to deficiencies in the induction protocol chosen for the experiment.

There are many plasticity events, each of them with their own mechanisms. This thesis may support the idea of a common target for, at least, most of them. Regardless of the mechanism for the expression of the plastic changes, p_v somehow seems to be involved. In all the events mentioned during this thesis (LTP, LTD, facilitation and PTP), p_v has been affected. Indeed, Short-Term Depression is in some cases explained by a reduction in p_v. Augmentation was also studied by our lab, although the data have not been included in this thesis because results were not so relevant for the main objectives.

Backing to the idea of Short-Term Plasticity as modulator or filter for Long-Term Plasticity, the theory of p_v as common target for plasticity reinforces the idea. A role for p_v seems hard to discard, although in many cases, it will be accompanied with another modifications. This may mean that the whole concept of plasticity has some basic statements that we have not defined yet. Even if it can still be classified according to the length of the expression or the direction of the changes, p_v might be a ubiquitous factor for the definition of plastic changes.

Conclusions

The presented study investigating the role of Syp family as well as p_v in plasticity yielded the following conclusions:

1. Syp family has not a direct role in the mechanism responsible for LTP, LTD or PTP.

2. The altered p_v caused by lack of Syp family affects to the expression of these synaptic events.

3. The expression of LTP depends on the p_v levels.

4. An enhanced p_v occludes PTP in both Schaffer collaterals and the calyx of Held.

5. Changes in the p_v due to modifications in the environmental Ca^{2+} concentration affect to the expression of LTP and PTP.

6. The changes in p_v due to lacking Syp family in QKO mice synapses can be influenced by changes in Ca^{2+} and plastic modifications.

7. Syp family act as regulator of the expression of synaptic potentiation.

Conclusiones

El presente estudio investigando los roles de la familia de Sinaptofisinas y el de la p_v en la plasticidad generó en las siguientes conclusiones:

1. La familia de Sinaptofisinas no tiene un rol directo en los mecanismos responsables de la LTP, LTD o PTP.

2. La p_v alterada debido a la ausencia de la familia de Sinaptofisinas afecta a la expresión de dichos mecanismos.

3. La expresión de la LTP depende de los niveles en la p_v.

4. Una p_v incrementada ocluye la PTP en las fibras colaterales de Schaffer y el caliz de Held.

5. Cambios en la p_v por modificaciones en la concentración extracelular de Ca^{2+} afectan a la expresión de la LTP y la PTP.

6. La p_v alterada debido a la ausencia de la familia de Sinaptofisinas en las sinapsis de ratones QKO puede verse afectada por cambios en la concentración de Ca^{2+} y por plasticidad.

7. La familia de Sinaptofisinas actúa como regulador de la expresión de la potenciación sináptica.

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