Presynaptic occlusion of long lasting NMDAR-dependent potentiation in Synaptophysin family knockouts

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Abstract

Synaptophysin family proteins are abundant components of synaptic vesicle membranes. Exocytosis is enhanced in knockouts, suggesting that family members are negative regulators of neurotransmission. Here we confirm previous reports of severe deficits in long-term potentiation (LTP) at Schaffer collateral synapses under experimental conditions that are standard for ex vivo experiments. However, LTP could be fully rescued by lowering extracellular Ca^{2+} enough to decrease baseline synaptic vesicle exocytosis to wildtype levels. Conversely, the deficit in LTP could be recapitulated at wildtype synapses by raising Ca^{2+} enough to increase baseline to knockout levels. Moreover, LTP induction was intact at both knockout and wildtype synapses under conditions where expression was blocked. The results indicate that the longest-lasting components of LTP expression are limited at a late stage in synaptic vesicle exocytosis from presynaptic terminals, and provide a straightforward new experimental strategy for dissecting apart mechanisms underlying induction and expression.

key words: LTP, Long-term potentiation, synaptophysin, Syp, synaptoporin, Snpr, Syp2, synaptogyrin, Sngr1, Sngr3

Introduction

Synaptophysin family proteins are ubiquitously expressed at high copy number within synaptic vesicle membranes; family members in mammalian synapses are Synaptophysin (Syp), Synaptoporin (Snpr), and Synaptogyrins 1 (Sngr1) and 3 (Sngr3) [1, 2]. Despite the abundance, Syp proteins are not required for high fidelity synaptic transmission. Instead, the *probability of release* of *readily releasable vesicles* - denoted with p_v - was elevated approximately 2-fold in quadruple knockout (QKO) synapses missing all four neuronal family members. Readily releasable vesicles are thought to be ready to release because they are docked to release sites embedded within the active zone region of the plasma membrane, and the elevated p_v observed at QKO synapses implies that the mechanism that catalyzes exocytosis is more efficient in the absence of Syp proteins. In contrast, no alterations were detected in other basic parameters of presynaptic function such as the number of readily releasable vesicles or the timing of vesicle recruitment to the *readily releasable pool* when vacancies arise [3]; vacancies are likely release sites that were *emptied* when the vesicle that had been docked underwent exocytosis.

These results and others where exocytosis was inhibited by heterologous expression of Syp or Sngr1 in PC12 cells suggest that endogenous Syp-family proteins function as negative regulators of the mechanism that catalyses exocytosis [4]. However, deficits

have been seen in both short- and long-term synaptic plasticity at Schaffer collateral synapses in double knockouts missing Syp and Sngr1 [5], and the relationship to regulation of exocytosis is not known.

Short- and long-term plasticity are both thought to be generated by multiple underlying mechanisms that can be distinguished by how long the alterations in synaptic strength are sustained. We confirmed previously that the most quickly decaying component of short-term plasticity - termed *facilitation*, and lasting only hundreds of milliseconds - was severely reduced at QKO synapses under standard conditions. We now show that the longest lasting component of long-term potentiation (LTP), lasting hours, is likewise severely reduced at QKO synapses.

Facilitation is induced and expressed cell autonomously, within individual presynaptic terminals. The precise mechanism of induction is still debated, but expression is widely thought to be manifest by an increase in neurotransmitter release from presynaptic terminals caused by an increase in p_v (e.g, [6]). And indeed: (a) facilitation at wildtype synapses is occluded by increasing extracellular Ca^{2+} [7], which elevates the baseline value of p_v by increasing the amount of Ca^{2+} flux into presynaptic terminals during action potentials [8–12]; and conversely, (b) facilitation at wildtype synapses is greatly increased simply by reducing extracellular Ca^{2+} [3], which relieves the occlusion by lowering the baseline p_v.

Lowering extracellular Ca^{2+} at QKO synapses *rescues* facilitation, indicating that Syp proteins do not play an essential role. Instead, the deficit seen at QKO synapses seems to be a secondary effect of elevating baseline p_v to a maximum/saturating level [7]; this is confirmed below by experiments showing that facilitation is the same at QKO and wildtype synapses when bathed in low extracellular Ca^{2+}.

Unlike facilitation, induction of LTP at Schaffer collateral synapses is not cell autonomous, but instead requires glutamate neurotransmitter release from presynaptic terminals, activation of NMDA-type glutamate receptors embedded within the membrane of postsynaptic compartments of separate neurons, and then second messenger signaling within the postsynaptic compartments [13]. Little evidence exists for postsynaptic expression of Syp proteins. However, one possibility that was debated extensively, but that never attained consensus status, is that some components of LTP might be expressed by presynaptic mechanisms that are activated by retrograde signals originating within the postsynaptic compartment [14–20]. If so, LTP expression might - like facilitation - involve elevating p_v, which might then be able to explain the reduction seen at QKO synapses [21, 22].

Here we provide strong evidence. First, we show that, like facilitation, LTP can be rescued at QKO synapses by lowering the extracellular Ca^{2+} from 2.0 to 1.2 mM, which lowers synaptic strength to wildtype levels. We then show that the deficit is at the level of expression because LTP induction was not prevented at QKO synapses in 2 mM Ca^{2+}. And, finally, elevating baseline synaptic strength at wildtype synapses by raising the extracellular Ca^{2+} occluded the expression of LTP without inhibiting induction.

Results

Facilitation is occluded at QKO synapses

We showed previously that the facilitation occurring after the second of two closely spaced pulses of stimulation - i.e., *paired-pulse facilitation* - is essentially absent at QKO Schaffer collateral synapses when extracellular Ca^{2+} is 2.6 mM, but is at least partly rescued by lowering Ca^{2+} to 1.8 mM. The results are reproduced in Supplementary Figure S1 along with previously unpublished results showing that paired-pulse facilitation is eliminated in the converse experiment at wildtype synapses where

extracellular Ca^{2+} was raised to high levels. However, a rigorous test of the hypothesis that the mechanism underlying facilitation is not altered at QKO synapses would require demonstrating that facilitation is the same at QKO and wildtype synapses when the baseline p_v is very low. We now report that this is indeed the case when extracellular Ca^{2+} is lowered to 0.5 mM (Fig. 1). These results support the hypothesis that the defect seen in paired-pulse facilitation at QKO synapses is a consequence of the elevated baseline p_v rather than a defect in the mechanism underlying facilitation.

The overall amount of facilitation during 10 Hz trains in 0.5 mM Ca^{2+} was >10-fold more than the paired pulse facilitation seen at wildtype synapses when Ca^{2+} was 2.6 mM. However, a large amount was expected in 0.5 mM Ca^{2+} because of previous studies in primary cell culture [23]. Similarly large amounts are not seen during train stimulation when Ca^{2+} is 2.6 mM both because of masking short-term depression caused by depletion of the readily releasable pool that is avoided when p_v is low, and because increases in p_v are occluded by a separate saturation mechanism [7].

Deficit in longest lasting phase of LTP

Facilitation was measured in the presence of 50 µM APV, which blocks NMDA receptors. In contrast, induction of LTP requires activation of postsynaptic NMDA receptors, and LTP experiments were therefore conducted in the absence of APV. We found that LTP was sustained as expected for at least 1 hr after induction at wildtype Schaffer collateral synapses when extracellular Ca^{2+} was 2 mM, but ran down almost completely at QKO synapses under the same conditions (Fig 2).

We used 2 mM Ca^{2+} rather than 2.6 mM to match conditions in a previous report of a deficit in LTP at Shaffer collateral synapses from Syp/Sngr1 double knockouts [5]. The new result broadly confirms the previous report. However, the reduced LTP in the previous report seemed to be stable for 1 hr post induction, whereas the LTP at QKO synapses in the present study declined over time (white lines in Fig 2a, quantified in Fig 2c). Nevertheless, even the earliest component of potentiation, observed immediately after induction, was probably less at QKO synapses (Supplementary Figure S3). The earliest component is sometimes termed STP, for short-term potentiation, but is blocked by NMDA-type glutamate receptor blockers (Supplementary Figure S4), and therefore, unlike facilitation, is likely not cell autonomous.

Rescue by lowering extracellular Ca^{2+}

The deficit in LTP at QKO synapses was rescued by lowering extracellular Ca^{2+} to 1.2 mM (Figure 3). The amount of LTP sustained for 1 hr was $39 \pm 7\%$ ($n = 14$) of pre-induction baseline at QKO synapses when bathed in 1.2 mM Ca^{2+}, which is similar to the value at wildtype synapses seen when Ca^{2+} was 2 mM ($35 \pm 7\%$; $n = 20$). In addition, the LTP rescued at QKO synapses by lowering extracellular Ca^{2+} was blocked by APV, confirming the requirement for NMDA receptors.

The LTP in Figures 2-3 was induced with two 1 s-long trains of 100 Hz stimulation separated by a 20 s rest interval, which is a widely used induction protocol, chosen to match the previous analysis of Syp/Sngr 1 double knockout synapses [5]. However, LTP continued to run down at QKO synapses after heavier stimulation that was previously shown to induce longer lasting LTP at wildtype synapses. And, the deficit was again rescued by lowering extracellular Ca^{2+} to 1.2 mM (Fig. 4). Follow-on experiments showed that LTP induced in 1.2 mM Ca^{2+} was similar at wildtype and QKO synapses, and the amount was near-maximal (Supplementary Figure S5). 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.

Intact expression

Next, we found that long lasting LTP could be unveiled at QKO synapses after inducing in 2 mM Ca^{2+} by subsequently lowering extracellular Ca^{2+} to 1.2 mM (Panel 5a and 1st bar in 5d). Interleaved control experiments: (a) confirmed that LTP ran down within an hour of induction at QKO synapses when Ca^{2+} was 2 mM (Fig 5b and 2nd bar of Fig 5d); and (b) showed that LTP was not induced in the absence of the 100 Hz stimulation (Fig 5c and 3rd bar of Fig 5d), which confirms that solution exchange from 2 mM Ca^{2+} to 1.2 mM was successful. These results indicate that the induction of LTP is not blocked at QKO synapses when Ca^{2+} is 2 mM, and consequently imply that the deficit seen when Ca^{2+} is 2 mM must be at the level of expression.

Lowering extracellular Ca^{2+} from 2 to 1.2 mM decreased baseline synaptic strength by a factor of 2.6 ± 0.1 at QKO synapses ($n = 15$; Fig. 5c), likely caused by an equivalent decrease in p_v [8, 10] to below baseline wildtype levels under standard conditions [3]. In contrast, lowering Ca^{2+} had no effect on the postsynaptic response measured from spontaneous miniature synaptic currents - i.e., "minis" - in CA1 principle neurons (Supplementary Figure S6), confirming that lowering Ca^{2+} lowered synaptic strength via a presynaptic mechanism. The results support the hypothesis that, like deficits in facilitation, deficits in LTP at QKO synapses are a consequence of the elevated baseline p_v rather than deficits in underlying mechanisms.

Occlusion of expression at wildtype synapses

If so, elevating the baseline synaptic strength at wildtype synapses to the baseline level at QKO synapses by increasing extracellular Ca^{2+} would similarly block the expression but not induction of LTP. This is confirmed by Figure 6.

That is, raising extracellular Ca^{2+} from 2 to 4 mM increased baseline synaptic strength by a factor of 2.4 ± 0.1 at wildtype synapses ($n = 26$), which is equivalent or more than the elevation seen at QKO compared to wildtype synapses under standard conditions [3]. Figure 6a and the 1st bar of Figure 6d confirm that LTP was blocked in 4 mM Ca^{2+}. Figure 6b and the 2nd bar of Figure 6d then show that the block is at the level of expression, not induction, because lowering Ca^{2+} back to 2 mM unveiled potentiation that was not expressed when Ca^{2+} was 4 mM. Finally, Figure 6c and the 3rd bar of 6d serve as a control showing solution exchange from 4 mM Ca^{2+} to 2 mM was successful. We included NMDA receptor blocker APV throughout the control for solution exchange because, otherwise, response sizes did not fully return to baseline, possibly because increased Ca^{2+} influx into the postsynaptic compartment via NMDA receptors may have been enough to induce a minor amount of LTP when the frequency of stimulation was low (Supplementary Figure S7; compare to Figure 6c and the 3rd bar of 6d). Finally, Panel b of Supplementary Figure S8 and bar 5 of Figure 6d confirm that neither LTP nor significant depression/run-down occurred in the absence of 100 Hz stimulation when measured in the continued presence of 4 mM Ca^{2+}.

Discussion

Here we show that the expression of the longest-lasting component of LTP is occluded at Schaffer collateral synapses by manipulations that elevate p_v, which is the probability with which readily releasable vesicles in presynaptic terminals undergo exocytosis following an action potential. In contrast, the mechanisms underlying induction of LTP are intact. The results provide a straightforward experimental method for disentangling mechanisms underlying LTP induction and expression by sequentially raising then lowering extracellular Ca^{2+}.

Indirect role for synaptophysin-family in LTP

We used two orthogonal manipulations to alter baseline p_v: (a) knocking out Syp-family proteins; and (b) altering extracellular Ca^{2+}. Knocking out Syp-family members elevates baseline p_v [3], and eliminates the later phase of LTP expression under standard conditions. Lowering Ca^{2+} after knocking out the Syp-family reverted baseline p_v to wildtype levels, and fully rescued expression whereas raising Ca^{2+} at wildtype synapses elevated baseline p_v and occluded expression. The results confirm an earlier report of deficits in LTP after knocking out two of the four Syp-family proteins [5], but indicate that Syp-family proteins do not play a direct role in underlying mechanisms. Instead, the deficits in LTP seen at QKO synapses are a consequence of the elevated baseline p_v.

Presynaptic mechanism for longest-lasting phase of LTP

It is widely agreed that LTP at Schaffer collateral synapses is induced by second messenger signaling within the postsynaptic cellular compartment - i.e., within *dendritic spines* - but no analogous consensus has yet emerged for the location of expression mechanisms [20]. The current results support the hypothesis that expression is at least partly via increased release of neurotransmitter from presynaptic terminals because: (a) Syp-family proteins are heavily expressed in presynaptic terminals; (b) p_v is a presynaptic parameter that directly influences the amount of neurotransmitter release; and (c) elevating baseline p_v by raising extracellular Ca^{2+} is already known to occlude components of short-term enhancement that are expressed as transient increases in p_v [7]. Conversely, the results argue against postsynaptic occlusion mechanisms as an explanation for the deficit at QKO synapses because: (a) lowering extracellular Ca^{2+} unveils LTP (Figures 3 - 5), but does not decrease postsynaptic sensitivity to glutamate release (Supplementary Figure S6); and (b) increasing extracellular Ca^{2+} occludes expression at wildtype synapses but does not increase postsynaptic sensitivity [11].

The new support for presynaptic expression complements previous studies showing: (a) more neurotransmitter release [14]; (b) more exocytosis from presynaptic terminals measured with FM1-43 dyes [17]; (c) mathematical analyses of changes in trial-to-trial fluctuations in synaptic responses measured with electrophysiological techniques [15, 16]; and, (d) optical measurements of increases in probability of release at individual synapses [18]. However: the conclusions from fluctuation analysis have been challenged, and a number of experiments indicated increases in postsynaptic expression of AMPA-type glutamate receptors without a corresponding increase in NMDA receptors, which would be difficult to explain by invoking presynaptic mechanisms [13].

One possibility is that LTP expression involves both pre- and postsynaptic mechanisms [20]. If so, the transient time course of maintenance seen at QKO synapses under standard conditions in Figures 2-5 suggests that postsynaptic mechanisms are predominantly expressed transiently, and then give way to long-lasting increases in p_v after 30-60 min, depending on the intensity of the induction protocol. Consistent with this, key evidence for postsynaptic expression is based on measurements made within 30 min of induction. This interpretation fits with the current concept that LTP expression occurs in at least three phases [24], each determined by separate underlying mechanisms.

Relationship between facilitation and LTP

The present results suggest that both facilitation and LTP are occluded by the elevated baseline p_v at QKO synapses, but the relationship between the mechanisms is not clear. There have been reports that LTP reduces facilitation [21, 22], suggesting shared

elements, but strong interactions between LTP and facilitation are not seen in all studies.

However, the absence of a strong interaction can be explained by our working model of presynaptic function. The model includes multiple classes of the release sites where synaptic vesicles dock to the active zone (Appendix of ref. [25]). The classes differ greatly in p_v. Most of the neurotransmitter released during low frequency stimulation - or after the first pulse of a closely spaced pair - is released by release sites with high p_v, which express paired-pulse depression rather than facilitation. In contrast, most of the neurotransmitter released after the second pulse of a pair is released by sites with low p_v, owing to strong facilitation. Because of this, our model predicts that decreases in facilitation caused by potentiating high p_v release sites would be offset by increases in facilitation caused by potentiating low p_v sites, and the amount of offset would depend strongly on experimental conditions.

Significance for memory

To our knowledge, the finding, here, that LTP expression can be occluded without simultaneously occluding induction is new, and may allow for memory reinforcement via a mechanism that preserves storage capacity. That is, precisely how memories are stored within neural circuits is not known, but the current concept is that individual memories are encoded by altering connection strength at many synapses, each of which additionally participates in unrelated memories. As a consequence, heavy reinforcement without any occlusion mechanism might interfere with memories implemented at overlapping populations of synapses. Meanwhile, an occlusion mechanism operating at the level of LTP induction might simply prevent reinforcement altogether. In contrast, the occlusion mechanism reported here operates at the level of expression where it might simultaneously reduce interference with other memories while allowing the synaptic changes underlying specific memories to persist longer after reinforcement.

Materials and Methods

QKO and matched wildtype mice were from the colony described in [3]. All experiments were conducted on 400 µm thick *ex vivo* transverse slices from hippocampus with CA3 removed as described in [11] except slices were incubated at 34 C for 30-60 min immediately after slicing and transferring to extracellular recording solution containing 2 mM Ca^{2+} and 2 mM Mg^{2+}. Extracellular recording solution additionally contained (in mM): 120 NaCl; 1.25 NaH_2PO_4; 26 $NaHCO_3$; 3.5 KCl; 10 glucose; 0.05 Picrotoxin. $MgCl_2$ was 2 mM except where indicated; $CaCl_2$ concentrations are as indicated in Results; Synaptic responses were recorded in whole cell voltage clamp mode by patch-clamping CA1 principle neurons for experiments in Fig. 1 and Supplementary Figures S1, S2, and S7, and as field potentials recorded in *stratum radiatum* for all others. Whole cell responses were measured as the current integral and field potentials were measured as the slope of the rising phase of the synaptic response between 15 and 85 % of peak. Intracellular recording solution for patch clamp experiments contained (in mM): 125 KGluconate; 8 NaCl; 10 Hepes; 1 EGTA; 10 KCl; 0.2 $CaCl_2$; 4 MgATP; 0.3 LiGTP, adjusted to pH 7.4 by adding KOH. Slices were from 13 - 21 day old mice (either sex) for patch-clamp recordings and from 6 - 8 week old males for field potential recordings. Responses were evoked with a $Ag/AgCl_2$ electrode inserted into a glass pipette with tip diameter ≥ 5 µm, filled with standard extracellular solution, and placed within *stratum radiatum* near the *stratum lacunosum-moleculare*. Stimulation was biphasic with intensity $\leq$ 100 µA for $\leq$ 100 µs.

Short-term enhancement in low Ca^{2+}: Stimulus intensity and microelectrode

placement were configured beforehand with extracellular 2 mM Ca^{2+} and 1 mM Mg^{2+}. Ca^{2+}/Mg^{2+} was then lowered to 0.5 mM/1.5 mM, which typically lowered response size to a point where some trials resulted in synaptic transmission failures. Stimulation artifacts were eliminated by subtracting the mean failure from all responses (see Supplementary Figure S2). Trials were repeated multiple times, and were averaged together after eliminating the stimulation artifact, but before further analysis. Criteria: Results from individual preparations were only included in the final analysis if the coefficient of variation of the 5 baseline responses after averaging across trials was less than 0.7, which eliminated preparations with excessive response-to-response scatter.

Declarations

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- Ethics approval All procedures were conducted in accordance with the 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).

- Authors' contributions: SdO and JJRG conducted experiments and contributed to writing. JFW provided mice in a blind fashion, designed experiments, analyzed the data, and wrote the manuscript.

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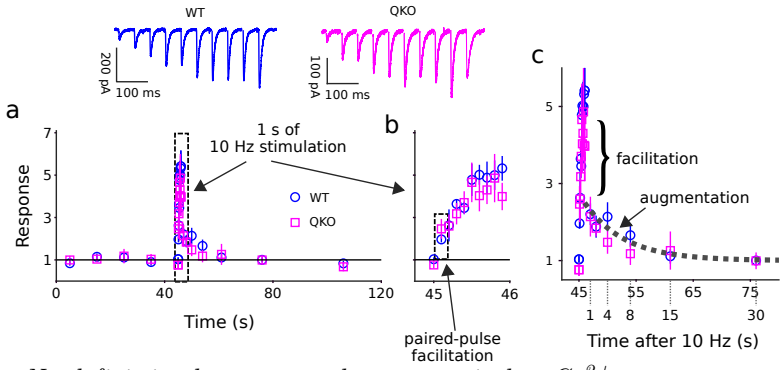


Figure 1. *No deficit in short-term enhancement in low Ca^{2+}.*
Experiments in 0.5 mM Ca^{2+}, 1.5 mM Mg^{2+}: Schaffer collaterals were stimulated 5 times, once every 10 s, to acquire a baseline, then with 10 pulses at 10 Hz to induce short-term enhancement, and finally with single pulses 1, 2, 4, 8, 15, 30, and 60 s after the end of 10 Hz stimulation (10 Hz stimulation included 11 instead of 10 pulses for some preparations). Trials were repeated multiple times per preparation and averaged together after eliminating the stimulus artifact before further analysis (see Supplementary Figure S2 and Methods for criteria). Responses were measured as the current integral then normalized by the mean baseline response.
(a) Mean response sizes during the entire experiment; $n = 5$ preparations for each genotype, each the mean of ≥ 9 trials. Traces at top are mean responses across all preparations during 10 Hz stimulation (i.e., after eliminating stimulus artifacts and averaging across trials).
(b) Replot of response sizes during 10 Hz stimulation on an expanded time scale. The 10 Hz train induced ~4.5-fold enhancement at wildtype and QKO synapses alike.
(c) Replot of response sizes after 10 Hz stimulation. The quickly decaying phase - complete in less than 1 s - is termed facilitation and the slowly decaying phase is termed augmentation [23, 26]. The theoretical curve is the single exponential $A \cdot e^{-t/\tau} + 1$ where $A = 1.5$ and $\tau = 7$ s, which is characteristic of augmentation.

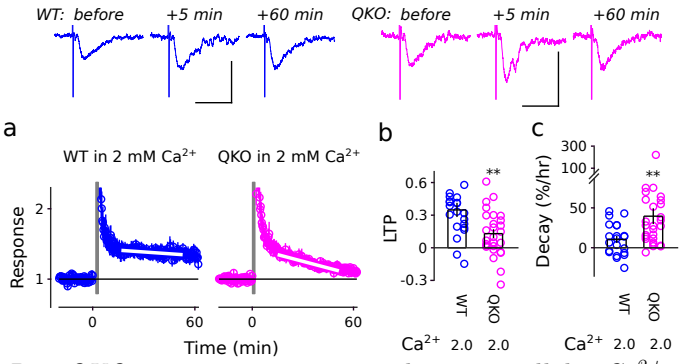


Figure 2. *LTP at QKO synapses is transient when extracellular Ca^{2+} is* 2 mM. Field potential responses were measured every 20 s before and after attempting to induce LTP with two 1 s-long trains of 100 Hz stimulation. Genotypes were interleaved. Experiments and analysis were blind to genotype. Inset are example traces before and after attempting to induce LTP. Scale bars are 0.5 mV by 20 ms.

(a) Mean response size over time, normalized by the mean baseline size during the 20 min prior to induction; induction was at Time = 0 (vertical gray bars). Error bars are s.e.m. ($n \geq 21$; see Methods for criteria). Note that y-axes are truncated; see Supplementary Figure S3.

(b) LTP was quantified as the mean minus baseline for minutes 51 - 60 after induction (** signifies $p < 0.01$, rank sum).

(c) Quantification of decay between minute 15 and 50 after induction, matching the white lines in (a).

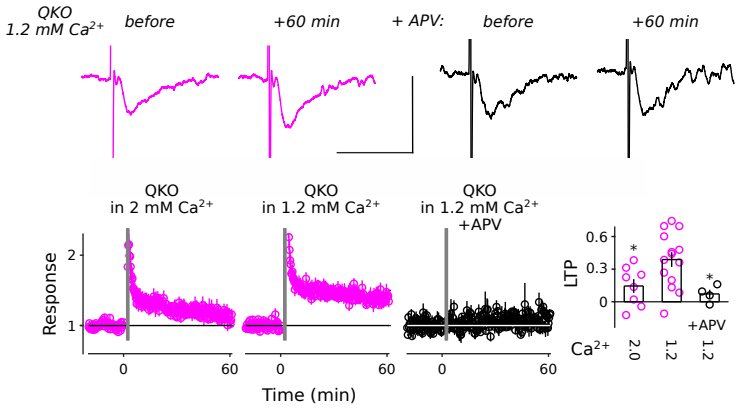


Figure 3. *LTP rescue and block by APV at QKO synapses when extracellular Ca^{2+} is* 1.2 mM.

LTP induction and other experimental details match Fig. 2, except the experimenter was not blind to experimental variables (* signifies $p < 0.05$, rank sum with Bonferroni correction for multiple comparisons, $n \geq 4$). Experimental variables were interleaved. Scale bars are 0.5 mV by 20 ms. The 2 mM Ca^{2+} experiments are not included in Fig. 2.

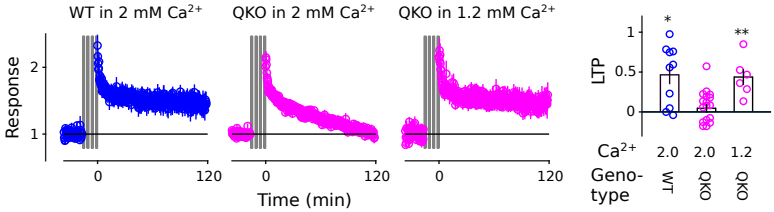


Figure 4. *LTP remains transient at QKO synapses after heavier induction when Ca^{2+} is* 2 mM.

LTP induction was 4 trains of 100 Hz stimulation separated by 5 min intervals (vertical gray bars). Wildtype and QKO experiments in 2 mM Ca^{2+} were interleaved, and the experimenter was blind to genotype, but the QKO experiments in 1.2 mM Ca^{2+} were done as a follow on, and was not blind. Analysis was blind to genotype and Ca^{2+} level. Scale bar in the inset is 1 mV by 20 ms.

(a) Response sizes versus time.

(b) LTP was quantified as the mean minus baseline for minutes $111-120$ after induction (* signifies $p < 0.05$, ** signifies $p < 0.01$, rank sum with correction for multiple comparisons, $n \geq 6$).

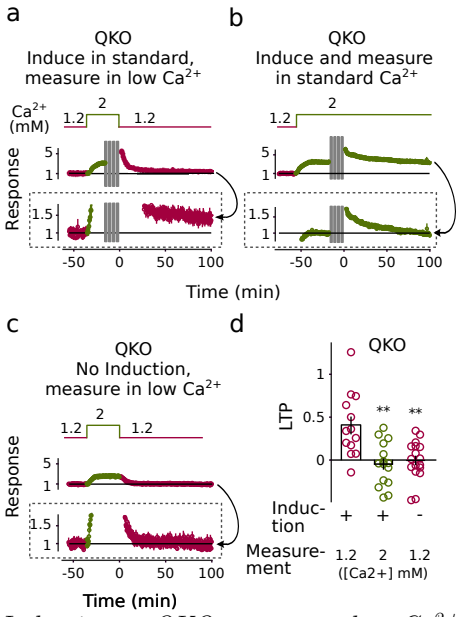


Figure 5. *Intact LTP Induction at QKO synapses when Ca^{2+} is* 2 mM.
For all experiments, baseline was first established when Ca^{2+} was 1.2 mM, and then again when 2 mM. LTP was induced with 4-trains of 100 Hz stimulation as in Fig. 4. Experiments were interleaved. The time needed to achieve baseline after changing to 2 mM Ca^{2+} varied between experiments, but this is omitted from the plots in **(a-c)**.
(a) Ca^{2+} was changed back to 1.2 mM after the LTP induction protocol. The boxed lower plot is the same data, except with an expanded y-scale to highlight the presence of substantial LTP.
(b) Ca^{2+} was maintained at 2 mM after the LTP induction protocol. The boxed lower plot is the same data, except re-normalized by the baseline recorded after changing to 2 mM Ca^{2+} to highlight the absence of LTP.
(c) Ca^{2+} was changed back to 1.2 mM without attempting to induce LTP. The boxed lower plot is the same data, except with an expanded y-scale to highlight that the successful change back to 2 mM Ca^{2+}.
(d) LTP was quantified as the mean minus baseline for minutes 91 - 100 after induction (** signifies $p < 0.01$, rank sum with correction for multiple comparisons, $n \geq 13$).

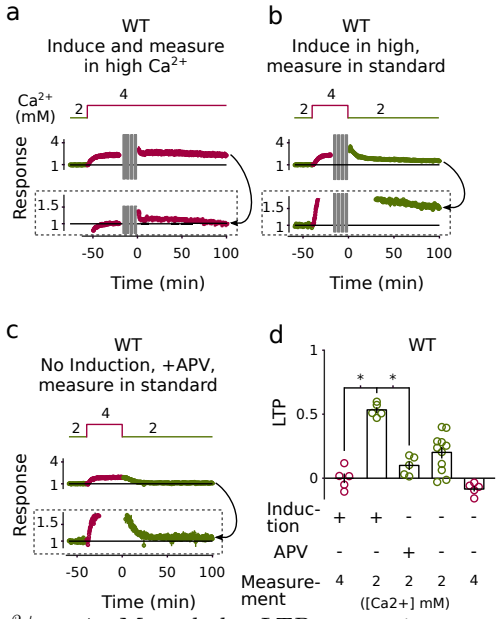
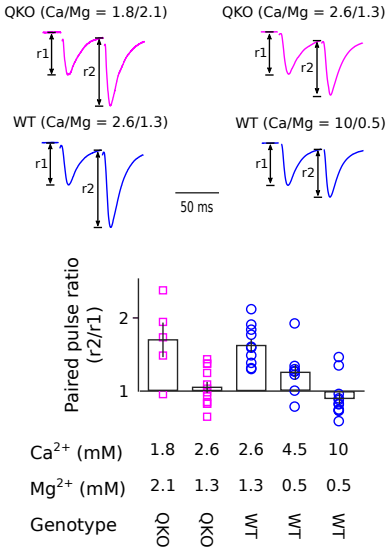
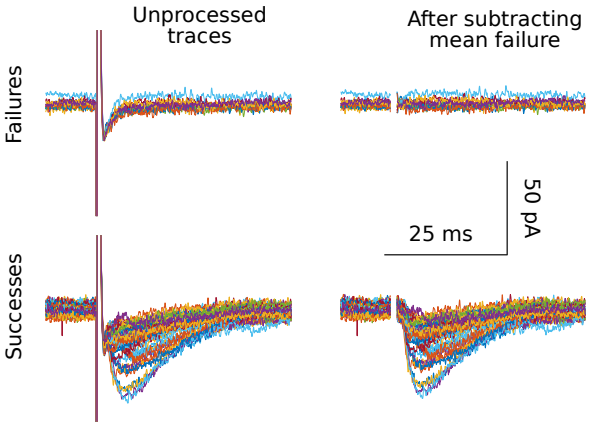


Figure 6. *Raising Ca^{2+} to* 4 mM *occludes LTP expression at wildtype synapses.*
For all experiments, baseline was first established when Ca^{2+} was 2 mM, and then again when 4 mM. LTP was induced with 4-trains of 100 Hz stimulation as in Fig. 4. The time needed to achieve baseline after changing to 2 mM Ca^{2+} varied between experiments, but this is omitted from the plots in **(a-c)**.
(a) Ca^{2+} was maintained at 4 mM after the LTP induction protocol. The boxed lower plot is the same data, except re-normalized by the baseline recorded after changing to 4 mM Ca^{2+} to highlight the absence of LTP.
(b) Ca^{2+} was changed back to 2 mM after the LTP induction protocol. The boxed lower plot is the same data, except with an expanded y-scale to highlight the presence of substantial LTP.
(c) Ca^{2+} was changed back to 2 mM without attempting to induce LTP. The boxed lower plot is the same data, except with an expanded y-scale to highlight that the successful change back to 2 mM Ca^{2+}. 50 µM APV was included throughout because the response size did not completely return to the baseline level without APV in previous experiments; these are documented in the 4th bar of **(d)** and in Supplementary Figure S7.
(d) LTP was quantified as the mean minus baseline for minutes $91 - 100$ after induction (* signifies $p < 0.05$, rank sum with correction for multiple comparisons; $n \geq 5$). See Supplementary Figures S7 - S8 for Response vs Time plots of the experiments documented in bars 4-5.

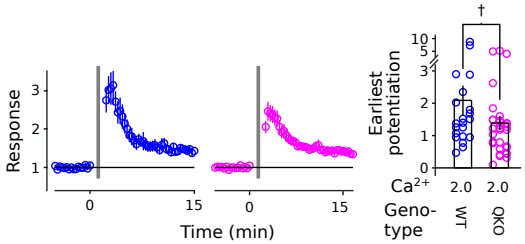
Experiments in **(a)** and **(b)** were interleaved with the experiments in Supplementary Figure S8 and approximately half of the experiments in Supplementary Figure S7, whereas experiments in **(c)** were follow on experiments undertaken when the response size did not fully reverse in the absence of LTP induction unless APV was included (compare 3rd and 4th bars in **(d)**).



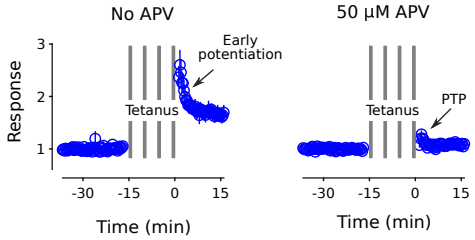
Supplementary Figure S1. Reduced paired-pulse facilitation at higher Ca^{2+} levels. The paired pulse ratio was measured by dividing the peak size of the second response during 20 Hz trains by the peak size of the first as indicated for the traces; traces are average of the first two responses across the entire data sets, and are scaled so that the first responses have the same amplitude. The first 3 bars and corresponding traces are reproduced from Figure 8 of ref. [3] and are included for comparison. Bars 4 and5 and corresponding traces are from previously unpublished data sets. The point is that it is possible to almost completely eliminate paired-pulse facilitation at wildtype synapses by increasing the extracellular Ca^{2+}, but a much higher level is needed compared to at QKO synapses.



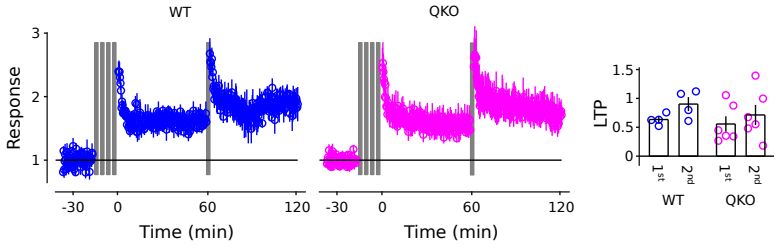
Supplementary Figure S2. *Procedure for eliminating stimulus artifacts in low Ca^{2+}* Leftmost traces are responses judged to be *Failures* (upper) and *Successes* (lower). Rightmost traces are after digitally subtracting the mean failure. The example is from a QKO preparation; Ca^{2+} was 0.5 mM and Mg^{2+} was 1.5 mM.



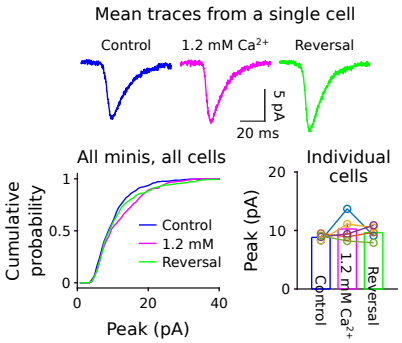
Supplementary Figure S3. *Earliest component of potentiation is probably reduced at QKO synapses* Same experiment as in Fig. 2, except truncated x-axis and full y-axis to highlight the full early component of potentiation. Earliest potentiation was estimated as the mean minus baseline of the first three data points immediately following induction († signifies $p = 0.057$, rank sum).



Supplementary Figure S4. *Earliest component of potentiation is NMDA receptor-dependent* The earliest component of potentiation was induced with 4 trains of 100 Hz stimulation as in Fig. 4 in the left panel, but not in the presence of NMDA receptor blocker APV. Data in the left panel are from the same data set as in the left panel of Fig. 4; $n = 10$ for the experiments in the presence of APV. The small component of early potentiation still present in the presence of APV is termed *post-tetanic potentiation* (PTP) [27].



Supplementary Figure S5. *Comparison of LTP at wildtype and QKO synapses in* 1.2 mM Ca^{2+}. LTP was first induced with 4 trains of 100 Hz stimulation as in Fig. 4 and then, after 60 min, with the standard 2 train protocol as in Fig. 2. LTP was quantified as the mean minus baseline for minutes 51 - 60 after the 1st induction protocol and for minutes 111 - 120 for the 2nd ($n \geq 4$).



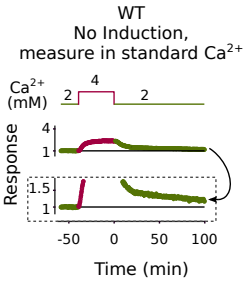
Supplementary Figure S6. *No change in size of minis when lowering Ca^{2+} to* 1.2 mM *at QKO synapses.*

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 % ($n = 5$ neurons).

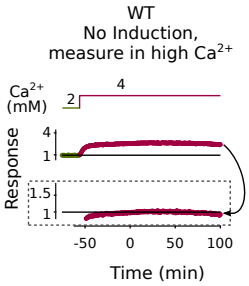
(a) Mean electrophysiological traces for a single neuron.

(b) Cumulative probability versus peak amplitude for all minis from all neurons.

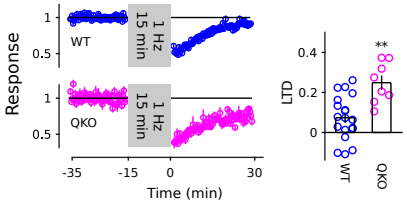
(c) Mean peak size of all minis from the individual neurons.



Supplementary Figure S7. *Response size vs time for 4th bar of Fig. 6d*



Supplementary Figure S8. *Response size vs time for* 5th *bar of Fig. 6d*



Supplementary Figure S9. *Increased long-term depression at QKO synapses.* Field potential responses were measured every 20 s before and after inducing LTD with 900 pulses of 1 Hz stimulation over 15 min, and quantified as the baseline minus mean for minutes 25 - 29 after induction ($n \geq 8$; ** signifies $p < 0.005$, rank sum). Extracellular Ca^{2+} was 2 mM.