Neocortical Layer-5 tLTD Relies on Non-Ionotropic Presynaptic NMDA Receptor Signaling

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Abstract

In the textbook view, NMDA receptors (NMDARs) act as coincidence detectors in Hebbian plasticity by fluxing Ca^{2+} when simultaneously depolarized and glutamate bound. Hebbian coincidence detection requires that NMDARs be located postsynaptically, but enigmatic presynaptic NMDARs (preNMDARs) also exist. It is known that preNMDARs regulate neurotransmitter release, but precisely how remains poorly understood. Emerging evidence suggest that NMDARs can also signal non-ionotropically, without the need for Ca^{2+} flux. At synapses between developing visual cortex layer-5 (L5) pyramidal cells (PCs), preNMDARs rely on Mg^{2+} and Rab3-interacting molecule 1αβ (RIM1αβ) to regulate evoked release during periods of high-frequency firing, but they signal non-ionotropically via c-Jun N-terminal kinase 2 (JNK2) to regulate spontaneous release regardless of frequency. At the same synapses, timing-dependent long-term depression (tLTD) depends on preNMDARs but not on frequency. We therefore tested if tLTD relies on non-ionotropic preNMDAR signaling. We found that tLTD at L5 PC→PC synapses was abolished by pre- but not postsynaptic NMDAR deletion, cementing the view that tLTD requires preNMDARs. In agreement with non-ionotropic NMDAR signaling, tLTD prevailed after channel pore blockade with MK-801, unlike tLTP. Homozygous RIM1αβ deletion did not affect tLTD, but wash-in of the JNK2 blocker SP600125 abolished tLTD. Consistent with a presynaptic need for JNK2, a peptide blocking the interaction between JNK2 and Syntaxin-1a (STX1a) abolished tLTD if loaded pre- but not postsynaptically, regardless of frequency. Finally, low-frequency tLTD was not blocked by the channel pore blocker MK-801, nor by 7-CK, a non-competitive NMDAR antagonist at the co-agonist site. We conclude that neocortical L5 PC→PC tLTD relies on non-ionotropic preNMDAR signaling via JNK2/STX1a. Our study brings closure to long-standing controversy surrounding preNMDARs and highlights how the textbook view of NMDARs as ionotropic coincidence detectors in plasticity needs to be reassessed.

Keywords

Long-term plasticity, STDP, LTD, LTP, NMDAR, unconventional NMDAR signaling

Introduction

Synapses continuously remodel in response to neuronal activity. Such synaptic plasticity is thought to underlie information storage (Bliss and Collingridge, 1993; Malenka and Bear, 2004; Nabavi et al., 2014) as well as developmental circuit refinement (Cline, 1998; Katz and Shatz, 1996; Song and Abbott, 2001), an idea often attributed to Donald Hebb (1949). More recent work tend to emphasize the role of temporal ordering of activity in determining plasticity, a notion called spike timing-dependent plasticity (STDP) (Markram et al., 2012).

In the STDP paradigm, coincident firing in the range of tens of milliseconds results in long-lasting changes in synaptic efficacy (Feldman, 2012). In classic STDP studies (Bi and Poo, 1998; Feldman, 2000; Markram et al., 1997; Zhang et al., 1998), presynaptic spikes leading postsynaptic spikes by a few milliseconds drive timing-dependent long-term potentiation (tLTP), whereas the opposite temporal ordering elicits timing-dependent long-term depression (tLTD). However, STDP is quite diverse, with rules and mechanisms depending on factors such as synapse type, developmental stage, and neuromodulation (Debanne and Inglebert, 2023; Larsen and Sjöström, 2015; McFarlan et al., 2023).

Many forms of STDP critically depend on NMDARs, which are a subfamily of glutamatergic receptors known for forming heterotetrameric ligand-gated ion channels (Paoletti et al., 2013; Wong et al., 2021). In the case of cortical tLTP, the role of NMDARs is well understood. Action potentials initiated at the soma are thought to backpropagate through dendrites (Stuart and Sakmann, 1994) to elicit nonlinear calcium signals localized to dendritic spines (Koester and Sakmann, 1998) by activating glutamate-bound NMDARs in the postsynapse (Yuste and Denk, 1995). These NMDARs are thus able to act as classic detectors of coincident EPSPs and action potentials in postsynaptic neurons (Markram et al., 1997) because of their dual need for presynaptically released glutamate as well as postsynaptic depolarization to relieve the Mg^{2+} blockade and flux the Ca^{2+} that triggers tLTP (Sjöström and Nelson, 2002; Wong et al., 2021).

In cortical tLTD, however, the role of NMDARs is not as well understood. We previously found evidence that, at L5 PC→PC connections, tLTD relies on preNMDAR signaling (Sjöström et al., 2003, 2007), but precisely how was not clear (Duguid and Sjöström, 2006). After all, the presynaptic spike is long gone by the time preNMDARs bind the released glutamate, arguing

that another presynaptic spike must arrive soon enough to depolarize and unblock glutamate-bound preNMDARs, suggesting a need for high-frequency spiking (Duguid and Sjöström, 2006).

More recently, we found that preNMDARs differentially regulate evoked and spontaneous release via RIM1aß and JNK2, respectively (Abrahamsson et al., 2017). Interestingly, the JNK-mediated regulation of spontaneous release by preNMDARs was not Mg^{2+}-sensitive (Abrahamsson et al., 2017), revealing the existence of an unconventional form of non-ionotropic NMDAR signaling in the axon (Bouvier et al., 2018; Dore et al., 2017; Wong et al., 2021). If tLTD relied on such non-ionotropic preNMDAR signaling, it would explain why it can be induced at both low and high frequencies, since in this signaling mode, preNMDARs are sensitivity to glutamate but not to Mg^{2+} or membrane potential.

Here, we explored how NMDARs signal in tLTD. We found that, regardless of frequency, L5 PC→PC tLTD relies on the non-ionotropic preNMDAR signaling pathway mediated by JNK2, which helps to resolve the long-standing enigma surrounding its lack of frequency dependence (Duguid and Sjöström, 2006).

Results

tLTD relies on presynaptically located NMDARs

We previously reported pharmacological and imaging evidence for preNMDARs at L5 PC→PC synapses that are necessary for neocortical tLTD (Abrahamsson et al., 2017; Buchanan et al., 2012; Sjöström et al., 2003, 2007), but their existence has been disputed (Christie and Jahr, 2009). We therefore wanted to directly challenge our previous findings by sparsely deleting the obligatory GluN1 NMDAR subunit (see Methods) and then measuring tLTD at synaptically connected L5 PC→PC pairs that lacked NMDARs pre- or postsynaptically.

With this approach, we found that preNMDAR deletion abolished high-frequency (HF) tLTD at 20 Hz (see Methods), whereas postsynaptic NMDAR (postNMDARs) deletion did not (**Figure 1**). We previously found that tLTD is expressed presynaptically (Sjöström et al., 2003, 2007). We relied on coefficient of variation (CV) analysis (Brock et al., 2020) to verify that postNMDAR deletion did not alter this (**Figure 1E**). We concluded that L5 PC→PC tLTD relied on pre- but not postNMDARs.

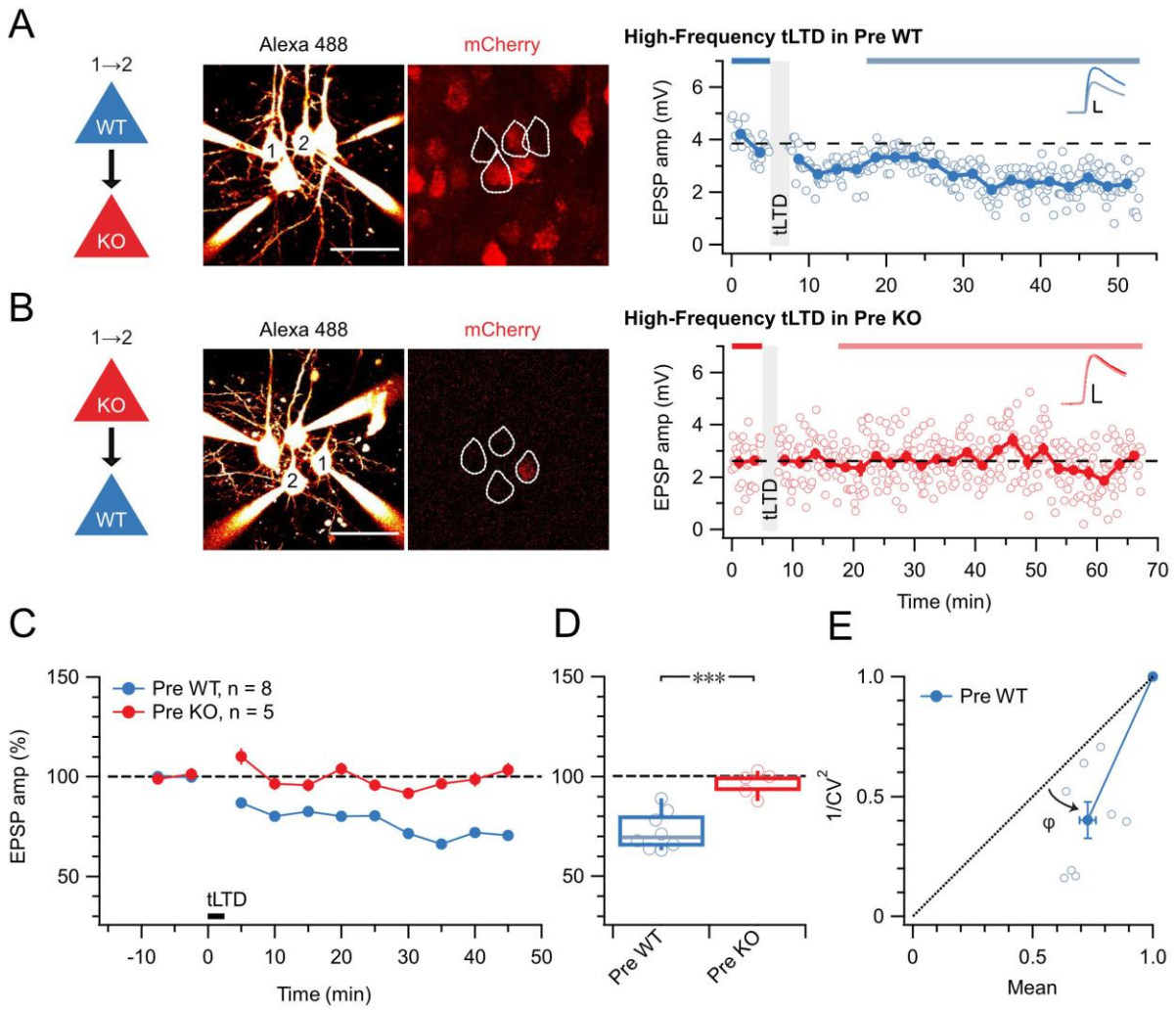

Figure 1. tLTD requires presynaptic NMDARs.

(A) Sample quadruple recording with L5 PCs visualized with Alexa 488 (left, scale bar 50 µm) and NMDAR deletion indicated by mCherry (right). In this wildtype (WT) →KO sample, high-frequency (HF) tLTD at 20 Hz (see Methods) was intact (after/before = 68%, p <0.001), suggesting postNMDARs were not required. Traces (right) were averaged over periods indicated by blue lines. Scale bars: 5 ms, 1 mV.

(**B**) For this KO→WT sample, however, the same induction elicited no plasticity (after/before = 100%, p = 0.99), suggesting tLTD needed preNMDARs. Scale bars as in A.

(C, D) HF tLTD was robustly evoked for presynaptic WT (Pre WT, blue) but not presynaptic KO pairs (Pre KO, red), verifying the need for preNMDARs in tLTD that we previously demonstrated (Sjöström et al., 2003, 2007). Pre WT: WT→WT and WT→KO pooled. Pre KO: KO→WT and KO→KO pooled.

(E) CV analysis indicated that tLTD was presynaptically expressed (φ = 19º ± 4º, n = 8, p < 0.001 vs. diagonal), in agreement with our prior studies (Sjöström et al., 2003, 2007).

Throughout this study, boxplots show medians and quartiles, with whiskers denoting extremes, but data is reported as mean ± SEM, with n indicating number of connections.

tLTD does not rely on ionotropic NMDAR signaling

We previously found that ionotropically signaling preNMDARs boost neurotransmitter release during periods of high-frequency activity at L5 PC→PC synapses and that this frequency dependence is inherited from the Mg^{2+} blockade of the preNMDAR channel (Abrahamsson et al., 2017; Wong et al., 2024). Since tLTD is not frequency dependent (Sjöström et al., 2003), we hypothesized that tLTD is not sensitive to blockade of the NMDAR channel pore. To test this hypothesis, we relied on the NMDAR pore blocker MK-801 (Song et al., 2018).

We first wanted to establish a positive control. MK-801 is known to block hippocampal LTP (Nabavi et al., 2013) as well as tLTP at excitatory inputs onto neocortical L2/3 PCs WT. At L5 PC→PC synapses, tLTP relies on different NMDARs than tLTD (Sjöström et al., 2003), but the MK-801 sensitivity has to our knowledge not been explored. Here, we found that MK-801 wash-in abolished potentiation (**Figure 2**), suggesting that L5 PC→PC tLTP relies on ionotropically signaling postNMDARs, like tLTP at other neocortical synapses (Bender et al., 2006; Nevian and Sakmann, 2006).

With this positive control established, we next explored if L5 PC→PC tLTD and tLTP were equally sensitive to MK-801. However, MK-801 had no effect on tLTD (**Figure 3**). Since the action of MK-801 is activity and voltage dependent (Huettner and Bean, 1988), we were concerned that HF tLTD and low-frequency (LF) tLTD (see Methods) might be differentially affected by this drug, but we found no difference (**Figure 3**).

Even with the positive tLTP control (**Figure 2**), we were concerned that the absence of effect of MK-801 on tLTD was a negative result (**Figure 3**). We therefore wish to strengthen our findings by complementing them with another approach. We thus attempted to block tLTD using 7-chlorokynurenate (7-CK), a co-agonist site blocker that abolishes NMDAR currents (Nabavi et al., 2013) without hindering glutamate binding (Kemp et al., 1988). However, 7-CK too had no effect on tLTD (**Figure 4**).

A parsimonious interpretation is that tLTP but not tLTD requires ionotropic NMDAR signaling. This interpretation additionally helps to explain why tLTD is not frequency dependent (Sjöström et al., 2003).

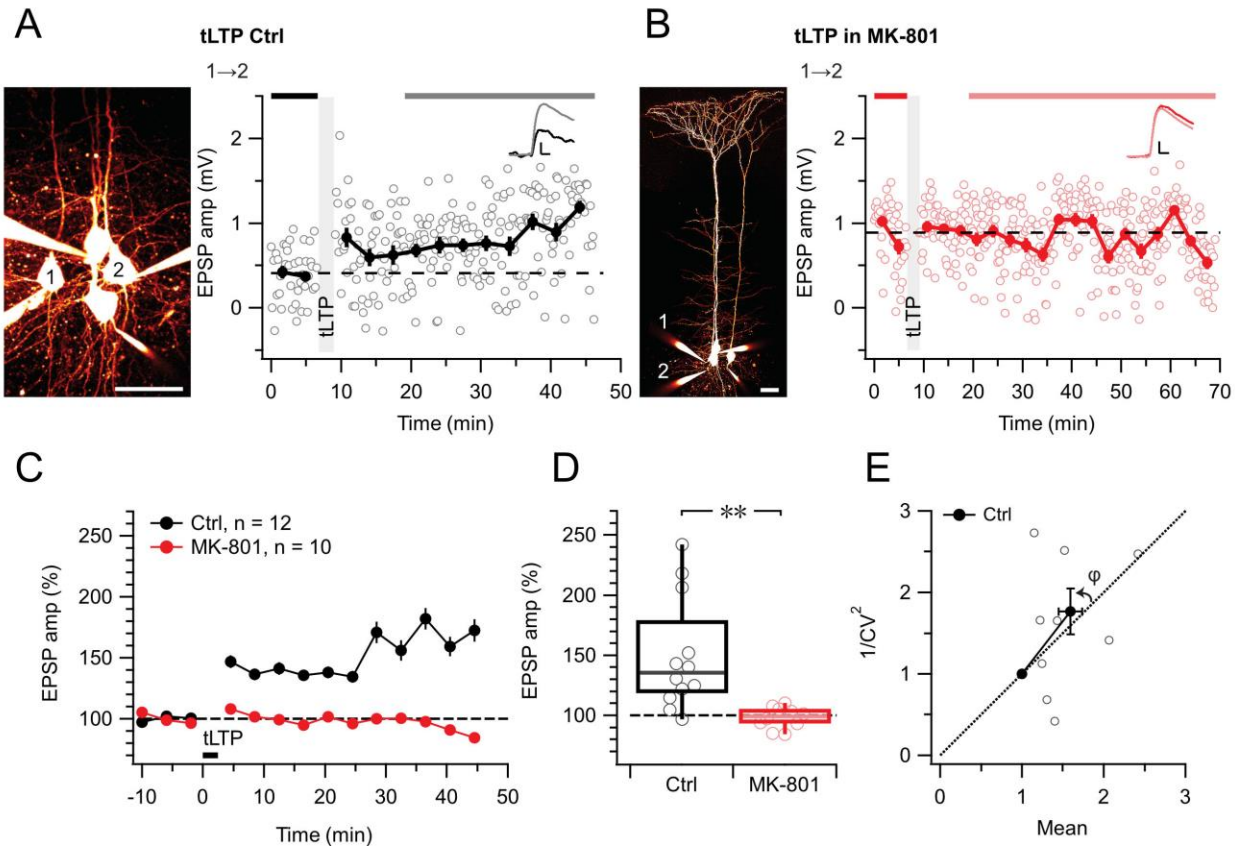

Figure 2. tLTP requires ionotropic NMDAR signaling.

(A) At this sample connection (PC 1→2, see Alexa 594 fills at left, scale bar: 50 µm), tLTP induction was successful (after/before = 210%, $p < 0.001$). Traces (right) were averaged over time periods indicated in black/grey. To avoid MK-801 affecting short-term depression (Abrahamsson et al., 2017; Sjöström et al., 2003), baseline spiking was 0.1 Hz. Scale bars: 5 ms, 0.2 mV.

(B) In this sample, MK-801 abolished tLTP with the same induction protocol (after/before = 95.44%, $p = 0.55$). Baseline spiking and scale bars as in (A).

(C, D) Ensemble data revealed that tLTP was robustly expressed in controls (black) but abolished in MK801 (red).

(E) CV analysis indicated that tLTP expressed both pre- and postsynaptically to varying degrees across different pairs ($\varphi = 11° \pm 15°$, $n = 10$, $p = 0.44$, two data points without potentiation were excluded, see Methods), in agreement with our prior work (Sjöström et al., 2007).

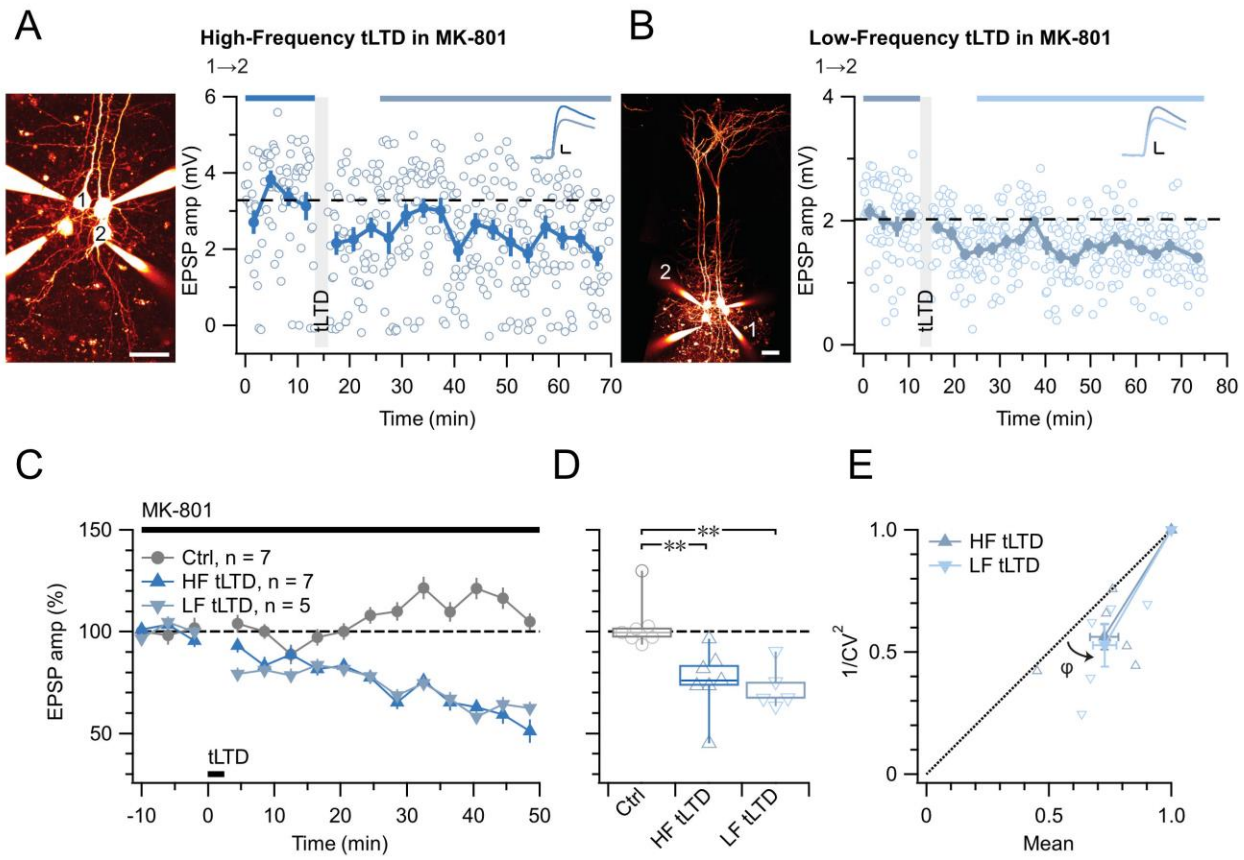

Figure 3. tLTD does not require ionotropic NMDAR signaling.

(A) At this sample connection (PC 1→2, see Alexa 594 fills left, scale bar: 50 µm), HF tLTD was successfully induced in the presence of MK-801 (after/before = 74%, $p < 0.001$). Traces were averaged over time periods indicated in blue/light blue. Baseline spiking was 0.1 Hz. Scale bars: 5 ms, 0.5 mV.

(B) In this sample, low-frequency (LF) tLTD at 1 Hz (see Methods) was successful in MK-801 (after/before = 78%, $p < 0.001$). Baseline spiking and scale bars as in A.

(C, D) Ensembles revealed robust tLTD at both low and high frequencies in MK-801 (blue triangles) compared to no-induction MK-801 controls (grey, Welch ANOVA $p < 0.01$), suggesting that tLTD does not rely on ionotropic NMDAR signaling.

(E) CV analysis indicated that tLTD at both induction frequencies was presynaptically expressed (HF LTD: $\varphi = 13^{o} \pm 5^{o}$, n = 6, $p < 0.05$; LF LTD: $15^{o} \pm 4^{o}$, n = 5, $p < 0.05$), in agreement with our prior findings (**Figure 1**) (Sjöström et al., 2003, 2007). One experiment with <5% plasticity was excluded, see Methods.

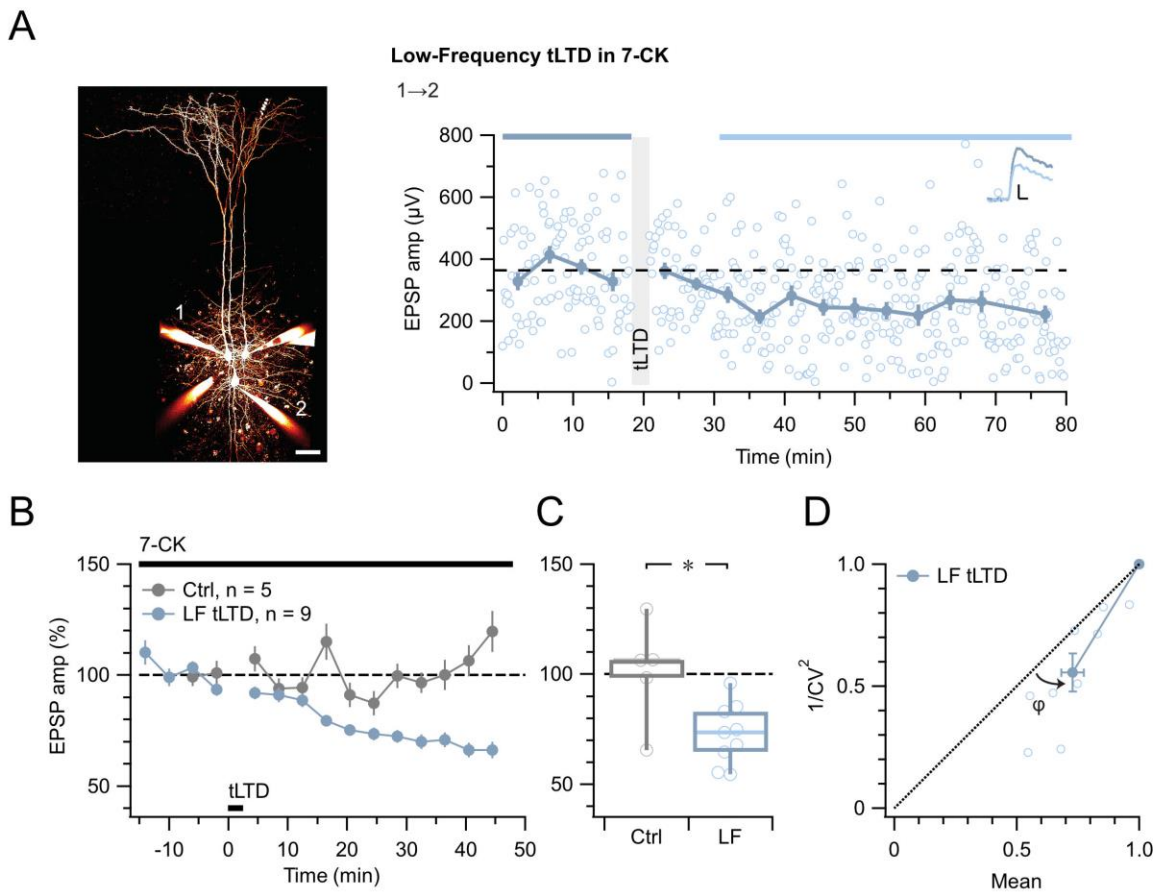

Figure 4. tLTD does not require ligand binding at NMDAR co-agonist site.

(A) At this sample connection (PC 1→2, see Alexa 594 fills at left, scale bar 50 µm), LF tLTD at 1 Hz (see Methods) persisted in the presence of 7-CK (after/before = 68 %, $p < 0.001$). Traces were averaged over time periods indicated in blue/light blue. Baseline spiking was 0.1 Hz. Scale bars: 5 ms, 0.1 mV.

(B, C) Ensemble revealed that LF tLTD persisted in 7-CK (blue) compared to no-induction 7-CK controls (grey), verifying that it does not require ionotropic NMDAR signaling.

(D) CV analysis verified that LF tLTD was presynaptically expressed ($\varphi = 14^{o} \pm 3^{o}$, n = 9; $p < 0.01$), in agreement with our prior findings (**Figure 1**, **Figure 3E**) (Sjöström et al., 2003, 2007).

tLTD relies on JNK2 but not on RIM1αβ

We previously found that ionotropic preNMDAR-mediated regulation of evoked release at L5 PC→PC synapses relies on RIM1αβ (Abrahamsson et al., 2017). Additionally, RIM1α is required for endocannabinoid-mediated LTD in hippocampus (Castillo et al., 2002; Chevaleyre et al., 2007) as well as for LTP in amygdala (Fourcaudot et al., 2008). We therefore explored if

RIM1αβ signaling similarly contributed to L5 PC→PC tLTD, by conditionally deleting RIM1αβ in excitatory neurons (see Methods and Abrahamsson et al., 2017).

We found that L5 PC→PC tLTD was robust in Emx1$^{Cre/+}$;RIM1αβ$^{fl/fl}$ animals (**Figure 5C-E**). The magnitude of tLTD in Emx1$^{Cre/+}$;RIM1αβ$^{fl/fl}$ mice was furthermore indistinguishable to that in WT mice (**Figure 5C, D**). To explore the locus of expression of tLTD, we analyzed paired-pulse ratio (PPR) and CV, which both suggested a presynaptic site of expression (**Figure 5E, F**), like we found before (**Figures 1**, **3E**, **4D**) (Sjöström et al., 2003, 2007). Homozygous RIM1αβ deletion thus had no detectable effect on L5 PC→PC tLTD.

Since we previously found that non-ionotropic preNMDAR signaling requires JNK2 to control spontaneous release (Abrahamsson et al., 2017), we were curious to test the potential need for JNK2 in tLTD. We blocked JNK2 signaling by pre-incubating (see Methods) with the specific inhibitor SP600125 (Bennett et al., 2001), which abolished tLTD (**Figure 5B-E**). Taken together, our results show that tLTD relies on JNK2 but not on RIM1αβ.

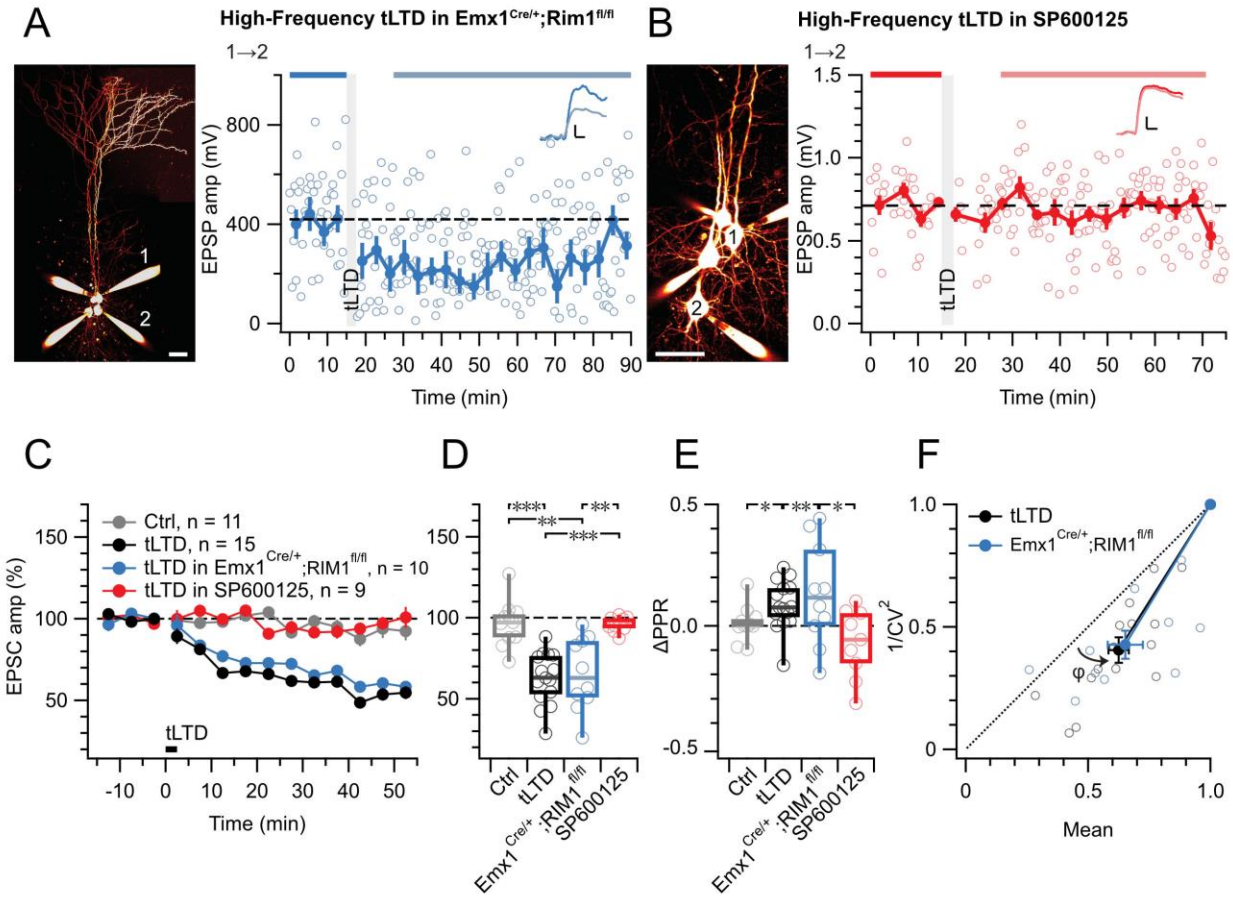

Figure 5. tLTD does not require RIM1αβ but relies on JNK2.

(A) At this sample PC 1→2 connection (left: Alexa 594 fills, scale bar 50 µm) in an acute slice from a homozygous RIM1αβ deletion mouse, tLTD persisted (after/before = 59%, $p < 0.001$). Scale bars: 5 ms, 0.1 mV.

(B) For this sample connection, the JNK2-blocker SP600125 (Bennett et al., 2001) abolished tLTD (after/before = 99%, $p = 0.86$). Scale bars: 5 ms, 0.2 mV.

(C, D) Ensemble data revealed that tLTD after homozygous RIM1αβ deletion (blue) was indistinguishable from WT tLTD (black), thereby dissociating tLTD from RIM1αβ. However, SP600125 robustly disrupted tLTD (red), with an outcome indistinguishable from no-induction controls (grey). Welch ANOVA $p < 0.001$.

(E) tLTD increased PPR (black and blue) compared to controls (grey), suggesting presynaptic expression. Welch ANOVA $p < 0.05$.

(F) CV analysis indicated that tLTD was presynaptically expressed, whether RIM1αβ was deleted ($\varphi = 16^{o} \pm 4^{o}$, $n = 10$, $p < 0.01$) or not ($\varphi = 13^{o} \pm 2^{o}$, $n = 15$, $p < 0.001$), in agreement with our prior findings (**Figure 1**, **Figure 3E**, **Figure 4D**) (Sjöström et al., 2003, 2007).

tLTD relies on presynaptic JNK2 signaling regardless of frequency

To selectively disrupt JNK2/STX1a interactions and associated signaling, we loaded a cell impermeable variant of the JGRi1 peptide (Marcelli et al., 2019) into pre- or postsynaptic PCs via the patch pipettes. When loaded presynaptically, HF tLTD was abolished, whereas when loaded postsynaptically, HF tLTD could be induced (**Figure 6A-D**). As before, tLTD was expressed presynaptically (**Figure 6E, F**). This suggested that tLTD was mediated by preNMDAR signaling via the JNK2/STX1a complex.

By comparison with preNMDAR regulation of spontaneous release via JNK2 — which was independent of Mg^{2+} and frequency (Abrahamsson et al., 2017) — this also suggested that the JGRi1 peptide ought to block tLTD at any frequency. We therefore repeated these JGRi1 peptide loading experiments for LF tLTD. As for HF tLTD, when the peptide was loaded presynaptically, LF tLTD was abolished, whereas when loaded postsynaptically, LF tLTD was expressed by presynaptic downregulation of release (**Figure 6G-L**). We note that at low frequency, preNMDARs remained blocked by Mg^{2+} (Abrahamsson et al., 2017; Wong et al., 2024), so this outcome lends further support to the view that tLTD requires non-ionotropic signaling. The sensitivity to pre- but not postsynaptic peptide further cements the view that this form of NMDAR signaling is located presynaptically.

Taken together, these results demonstrate that, regardless of induction frequency, tLTD is mediated by non-ionotropic preNMDAR signaling via the JNK2/STX1a complex in presynaptic neurons.

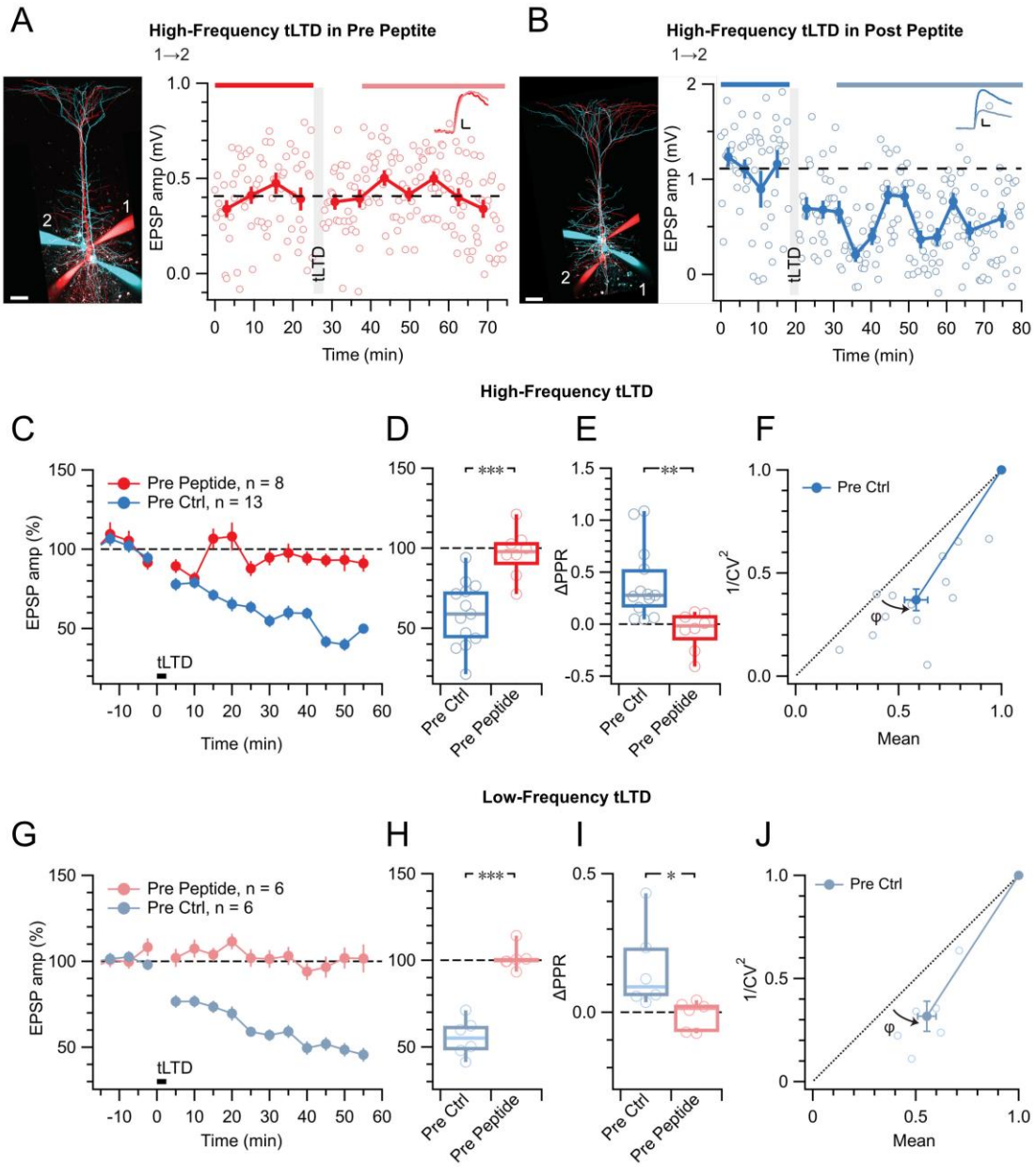

Figure 6. tLTD relies on presynaptic JNK2/STX1 signaling.

(A) Presynaptic loading of peptide disrupting JNK2/STX1 interactions (Marcelli et al., 2019) in this PC 1→2 pair abolished HF tLTD (after/before = 105%, p =0.52). Red cells were loaded with peptide, whereas blue cells were not. Traces were averaged over time periods indicated in red/pink. Scale bar 50 µm, insert scale bars 5 ms, 0.1 mV.

(B) Postsynaptic peptide loading in this PC 1→2 pair had no effect on HF tLTD (after/before = 47%, $p < 0.001$). Insert scale bars 5 ms, 0.2 mV.

(C, D) Presynaptic (red) but not postsynaptic/no peptide loading (blue) abolished HF tLTD, demonstrating a need for presynaptic JNK2/STX1 signaling.

(E) Without presynaptic peptide (blue), HF tLTD induction increased PPR as expected from reduced release, whereas with presynaptic peptide (red), PPR expectedly remained unaffected.

(F) CV analysis of HF tLTD with no presynaptic peptide agreed with presynaptic expression ($\varphi = 13^{o} \pm 3^{o}$, $n = 13$, $p < 0.001$).

(G, H) Presynaptic (red) but not postsynaptic/no peptide loading (blue) abolished LF tLTD, demonstrating that the need for presynaptic JNK2/STX1 signaling was not frequency dependent like ionotropic preNMDAR signaling is (Abrahamsson et al., 2017).

(I) Without presynaptic peptide (blue), LF tLTD induction increased PPR in keeping with presynaptic expression, but with presynaptic peptide (red), PPR was unaffected.

(J) CV analysis of LF tLTD with no presynaptic peptide agreed that release was reduced ($\varphi = 11^{o} \pm 2^{o}$, $n = 6$, $p < 0.01$).

Discussion

The role of preNMDARs in tLTD has long been enigmatic (Duguid and Sjöström, 2006; Sjöström et al., 2003). Our study provides resolution to this long-standing enigma by showing for the first time that tLTD relies on non-ionotropic preNMDAR signaling via JNK2. Our findings thereby further challenge the traditional view of NMDA receptors as ionotropic coincidence detectors and reveal how non-ionotropic preNMDAR signaling can shape STDP.

Reconciling decades of preNMDAR controversy

Although evidence for preNMDARs have been reported for decades (Aoki et al., 1994; Berretta and Jones, 1996; DeBiasi et al., 1996; Glitsch and Marty, 1999; Liu et al., 1997), this receptor type has been controversial. For instance, one study (Christie and Jahr, 2009) could not find the preNMDARs that we reported as underlying L5 PC→PC tLTD (Sjöström et al., 2003, 2007). Several other studies, however, largely corroborated our findings of preNMDARs at L4 PC → L2/3 PC synapses and elsewhere (Banerjee et al., 2014; Banerjee et al., 2009; Bender et al., 2006; Brasier and Feldman, 2008; Corlew et al., 2007; Rodríguez-Moreno and Paulsen, 2008). Then again, one study argued that the NMDARs that underlie L4 PC → L2/3 PC tLTD are actually postsynaptic (Carter and Jahr, 2016). Similar disagreements exist in cerebellum, with some studies reporting preNMDARs (Casado et al., 2000; Casado et al., 2002; Glitsch and Marty, 1999) and others arguing that they do not exist (Christie and Jahr, 2008).

Central to the preNMDAR controversy has been how they signal. Classically, NMDARs need both glutamate and depolarization to relieve Mg^{2+} blockade, open up, and signal via calcium flux (Dore et al., 2017; Wong et al., 2021). Consistently, preNMDARs often require high-frequency spike trains to activate (Abrahamsson et al., 2017; Bidoret et al., 2009; Buchanan et al., 2012; McGuinness et al., 2010; Sjöström et al., 2003; Wong et al., 2021), as a single spike that elicits glutamate release is gone by the time preNMDARs bind the glutamate. However, preNMDARs also govern spontaneous release (Abrahamsson et al., 2017; Berretta and Jones, 1996; Buchanan et al., 2012; Kunz et al., 2013; Sjöström et al., 2003) and tLTD (Sjöström et al., 2003), which both occur at low frequencies, which lead to a long-standing contradiction in the field (Banerjee et al., 2009; Duguid and Sjöström, 2006; Sjöström et al., 2003).

This controversy can be resolved by several intriguing preNMDAR properties. Whereas postNMDARs are ubiquitously expressed, preNMDARs are only found at specific synapse types (Banerjee et al., 2014; Brasier and Feldman, 2008; Buchanan et al., 2012; Larsen et al., 2014). PreNMDARs are thus easy to miss if different synapse types are mixed experimentally. Furthermore, preNMDARs often rely on particular subunits and conditions that reduce the flux of Ca^{2+} (Banerjee et al., 2009; Kunz et al., 2013; Larsen et al., 2011), leading to weak signals that may be hard to detect.

Finally, preNMDARs can also signal non-ionotropically to control release (Abrahamsson et al., 2017; Wong et al., 2024). If the expectation is that NMDARs can only signal ionotropically, this may confound experimental interpretation. For instance, manipulations such as loading cells internally with MK-801 to block NMDARs from the inside or with BAPTA to chelate Ca^{2+} may become inconclusive. However, non-ionotropic NMDAR signaling has been reported broadly, for instance in classic LTD (Nabavi et al., 2013; Nielsen et al., 2024; Park et al., 2022), structural plasticity (Park et al., 2022; Stein et al., 2021; Thomazeau et al., 2021), and ischemic excitotoxicity (Weilinger et al., 2016), so this mode of NMDAR signaling is not necessarily rare.

In fact, non-ionotropic signaling has also been found for other ionotropic channels, such as kainate receptors (Rodriguez-Moreno and Lerma, 1998), AMPA receptors (Takago et al., 2005; Wang and Durkin, 1995), and even voltage-gated Ca^{2+} channels (Trus and Atlas, 2024). The

classic textbook view of receptor types as strictly ionotropic or non-ionotropic is therefore not solidly anchored in the biology.

Finally, our finding that preNMDAR signaling in L5 PC→PC tLTD is non-ionotropic explains why this form of plasticity does not depend on frequency (Duguid and Sjöström, 2006; Sjöström et al., 2003). This is because, in this signaling mode, preNMDARs are sensitivity to glutamate but not to Mg^{2+} or membrane potential, so there is no need for spikes to arrive in quick succession to provide the depolarization that would be required for ionotropic preNMDAR signaling (Duguid and Sjöström, 2006).

Overall, our finding that non-ionotropically signaling preNMDARs are key to L5 PC→PC tLTD thus align with much of the prior literature while simultaneously bringing closure to long-standing controversies (Bouvier et al., 2018). Our genetic deletion approach furthermore firmly situate the NMDARs needed for tLTD in the pre- rather than the postsynaptic cell of L5 PC→PC pairs (Carter and Jahr, 2016).

A hitherto unappreciated role for JNK2 in tLTD

JNKs are Serine-Threonine kinases belong to the mitogen-activated protein kinase family and mediate stress stimuli, e.g., cytokines, ultraviolet irradiation, and heat shock. There are three closely related vertebrate genes: JNK1 and JNK2 are ubiquitous, while JNK3 is primarily neuronal (Yamasaki et al., 2012). In the brain, JNKs are key developmental regulators, for instance of neuronal migration, dendrite formation, and axon maintenance (Yamasaki et al., 2012).

It has long been known that JNK is activated by NMDA (Coffey, 2014; Mukherjee et al., 1999). More recently, we and others established that preNMDARs regulate spontaneous release by signaling via JNK2 (Abrahamsson et al., 2017; Nisticò et al., 2015) and that this regulation critically depends on an interaction between JNK2 and STX1a (Marcelli et al., 2019). Interestingly, it was previously proposed that JNK2 signaling is involved in behavioral learning as well as in classical NMDAR-dependent LTD (Curran et al., 2003; Morel et al., 2018). Our study, however, is to our knowledge first to show a need for JNK2 signaling specifically in tLTD, which relies on mechanistic underpinnings that differ from classical LTD, such as retrograde endocannabinoid signaling (Bender et al., 2006; Nevian and Sakmann, 2006; Sjöström

et al., 2003). Our present study thus extends the prior literature by suggesting that JNK2 signaling may be a general principle that applies to distinct forms of long-term plasticity.

Diverse preNMDAR signaling across different synapse types

In our hands, the NMDAR channel pore blocker MK801 surprisingly did not block tLTD at L5 PC→PC connections, even though several other studies of tLTD at L4 PC → L2/3 PC synapses reported that MK801 abolishes tLTD (Banerjee et al., 2014; Larsen et al., 2014; Rodríguez-Moreno et al., 2013; Rodríguez-Moreno et al., 2011; Rodríguez-Moreno and Paulsen, 2008). This difference is likely due to non-ionotropic versus ionotropic preNMDAR tLTD at L5 PC→PC and L4 PC → L2/3 PC synapses, respectively. This mechanistic distinction is generally consistent with the view that STDP depends on synapse type (Larsen and Sjöström, 2015; McFarlan et al., 2023). In fact, tLTD is mediated by distinct mechanisms even for different synaptic input types onto the same L2/3 PCs (Banerjee et al., 2009; Larsen et al., 2014).

A corollary from this synapse-specific difference in non-ionotropic versus ionotropic preNMDAR tLTD at L5 PC→PC and L4 PC → L2/3 PC synapses is a differential frequency dependence. Indeed, at L4 PC → L2/3 PC synapses, a frequency-dependent form of presynaptic self-depression has been reported (Rodríguez-Moreno et al., 2013). Such frequency dependence of tLTD does not, however, exist at L5 PC→PC connections (Sjöström et al., 2003).

Curiously, preNMDARs at L5 PC→PC connections signal ionotropically to boost neurotransmitter release during high-frequency firing (Abrahamsson et al., 2017; Buchanan et al., 2012; Sjöström et al., 2003; Wong et al., 2024). This boosting relies on RIM1αβ (Abrahamsson et al., 2017) and mTOR-mediated protein synthesis in L5 PC axons (Wong et al., 2024), yet is synapse-type-specific, so does not affect L5 PC→interneuron synapses (Buchanan et al., 2012; Wong et al., 2024). Consequently, MK801 blocks preNMDAR-mediated boosting of evoked L5 PC→PC release (Abrahamsson et al., 2017; Buchanan et al., 2012; Sjöström et al., 2003).

Another corollary from non-ionotropic preNMDAR signaling in tLTD is that removing Mg^{2+} to unblock the channel pore should not induce LTD at L5 PC→PC synapses during low-frequency firing. In agreement, reduced Mg^{2+} does not elicit LTD but actually boosts neurotransmission (Abrahamsson et al., 2017; Wong et al., 2024). Whether or not Mg^{2+} washout promotes LTD at L4 PC → L2/3 synapses has not been explored, as far as we know.

Caveats

We relied on JNK2 blockade as a proxy for non-ionotropic preNMDAR signaling, as we established this hallmark feature of flux-independent preNMDAR signaling of L5 PCs in a previous study (Abrahamsson et al., 2017). It is, however, presently unclear how well this proxy generalizes. A drug that specifically blocks non-ionotropic but not ionotropic NMDAR signaling would resolve this, much like MK801 does the vice versa. To our knowledge, such a drug presently does not exist.

Although tLTD at L5 PC→PC synapses does not depend on frequency (Sjöström et al., 2003), consistent with the non-ionotropic preNMDAR signaling we provide evidence for here, chemical LTD (cLTD) induced by wash-in of cannabinoid CB1 receptor antagonists is in fact frequency dependent (Sjöström et al., 2003). This apparent discrepancy is difficult to explain, because tLTD requires CB1 receptor signaling at low as well as high frequencies (Sjöström et al., 2003). Although the frequency dependence of cLTD is consistent with its known sensitivity to MK801 (Sjöström et al., 2003), an apparent disagreement remains. A potential explanation is that CB1 receptor signaling in tLTD is phasic whereas that in cLTD is tonic, and phasic/tonic CB1 signaling paths are known to differ mechanistically (Castillo et al., 2012). To address this, rapid photolysis of caged CB1 receptor agonists (Heinbockel et al., 2005) may mimic tLTD better than slow wash-in of cannabinoid CB1 receptor antagonists (Sjöström et al., 2003). Resolving this enigma will require future work.

Future directions and implications for disease

Our study provides fresh perspective on non-ionotropic function for preNMDARs in tLTD at neocortical synapses. By engaging JNK2-mediated signaling independent of Ca^{2+} influx, preNMDARs contribute to STDP in a manner that has not been previously appreciated. Our findings challenge the traditional view of NMDARs as coincidence detectors in Hebbian plasticity and highlight the diversity of synaptic plasticity mechanisms (Larsen and Sjöström, 2015; McFarlan et al., 2023). Future studies will be needed to explore the broader implications of non-ionotropic NMDAR signaling in other brain regions and under different physiological conditions.

It is crucial to understand how NMDARs operate, as they are hotspots for major synaptic pathologies such as Alzheimer, schizophrenia, and epilepsy (Nugent et al., 2022; Paoletti et al., 2013). For instance, a central hypothesis in schizophrenia research is based on NMDAR hypofunction (Lisman et al., 2008). Yet, if one were to rationally create an NMDAR-based therapy for schizophrenia, one would need to know which type of NMDAR signaling is relevant. Future studies may thus reveal the potential therapeutic relevance of targeting distinct NMDARs signaling pathways.

Methods

Animals and Ethics Statement

The animal study was reviewed and approved by the Montreal General Hospital Facility Animal Care Committee (The MGH FACC) and adhered to the guidelines of the Canadian Council on Animal Care (CCAC). At postnatal day 11 to 18, male or female mice were anaesthetized with isoflurane and sacrificed once the hind-limb withdrawal reflex was lost. Transgenic animals had no abnormal phenotype. Sparse NMDAR deletion was achieved by removing the obligatory Grin1 subunit in a subset of L5 PCs by neonatal injection (Kim et al., 2014) of AAV-eSYN-mCherry-iCre into V1 of Grin1$^{fl/fl}$ animals obtained from The Jackson Laboratory (#005246, see below). A Cre-loxP recombinase strategy (Nagy, 2000) was used to generate transgenic mice after two generations with RIM1αβ homozygously conditionally deleted in excitatory cells, as genome-wide RIM1αβ knockout impairs survival (Mittelstaedt et al., 2010). Homozygous Emx1$^{Cre/Cre}$ mice (Gorski et al., 2002) were obtained from The Jackson Laboratory (#005628). Homozygous RIM1αβ$^{fl/fl}$ mice (Kaeser et al., 2008) were kindly gifted by Pascal Kaeser (Harvard University, MA). Heterozygous Emx1$^{Cre/+;}$RIM1αβ$^{fl/+}$ mice were generated by crossing Emx1$^{Cre/Cre}$ with RIM1αβ$^{fl/fl}$ mice. Emx1$^{Cre/+}$;RIM1αβ$^{+/+}$ and RIM1αβ$^{fl/fl}$ mice were generated by crossing Emx1$^{Cre/Cre}$;RIM1αβ$^{fl/+}$ mice with RIM1αβ$^{fl/+}$;no-Cre mice (Abrahamsson et al., 2017). These distributed in a Mendelian fashion and had viability indistinguishable from that of C57BL/6 mice. To determine the genotype of each animal, tail biopsy and tattooing were performed on mice before the age of P6. Genotyping was carried out using standard methodology with Jackson Laboratory primers (RIM1: 12061, 12062; Emx1: oIMR1084, oIMR1085, oIMR4170, oIMR4171) using QIAGEN HotStarTaq DNA Polymerase kit (203203) and dNTPs from Invitrogen/Thermo-Fisher (18427-013) (Abrahamsson et al.,

2017). Wildtype (WT) denotes C57BL/6J (Jackson Laboratory #000664), RIM1αβ$^{fl/fl}$;no-Cre and genetically unaffected littermates (Emx1$^{Cre/+}$;RIM1αβ$^{+/+}$).

Viral injections

Cre recombinase was delivered by viral injection of AAV9-eSYN-mCherry-T2A-iCre-WPRE (Vector Biolabs, Cat No. VB4856) into the primary visual cortex of Grin1$^{fl/fl}$ neonates (P0-2) to generate a conditional NMDR deletion through Cre-loxP recombination at the site of the Grin1 gene. This cuts the Grin1 genetic sequence thus preventing the cell from producing the GluN1 subunit. As the GluN1 subunit is obligate, the cells expressing the virus will not express functional NMDARs. Cells expressing the viral construct were detected via an mCherry tag. Pups were anesthetized by putting them on ice and viral injection was delivered with a needle syringe held in a stereotactic injection setup and connected to a microinjector apparatus. The animal head was held in place with ear bars and the tip of the injection needle was zeroed to lambda. The needle was then positioned to the following coordinates: x = ± 1.10; y = 0.00. The needle was lowered until it reached the pial surface, where the z coordinate was zeroed. Three injections of 0.2-0.3µl each were performed at z1 = -0.20; z2 = -0.15; and z3 = -0.10. Both hemispheres were injected to increase the number of slices available for experiments and to reduce the risk of seeing no expression in an animal on the experimental day, both of which enhance productivity. The AAV9 serotype has a particularly high tropism for the central nervous system (Foust et al., 2009) and the enhanced synapsin (eSYN) promoter specifically targets neurons (Hioki et al., 2007). T2A is a self-cleaving peptide and facilitates co-expression of Cre recombinase and mCherry (Liu et al., 2017). Finally, the Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element (WPRE) enhances expression levels of the viral-encoded proteins (Klein et al., 2006), allowing successful expression in neocortical neurons, including pyramidal cells. By controlling the viral titer, expression levels were regulated to achieve sparse genetic deletion of NMDARs in primary visual cortex neurons (Kim et al., 2014).

Acute Slice Preparation

After decapitation, the brain was removed and placed in ice-cold (~4°C) artificial cerebrospinal fluid (ACSF), containing in mM: 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 26 $NaHCO_3$ and 25 glucose, bubbled with 95% O_2/5% CO_2. Osmolarity of the ACSF was adjusted to 338 mOsm with glucose. Oblique coronal 300-µm-thick acute brain slices were

prepared using a Campden Instruments 5000 mz-2 vibratome (Lafayette Instrument, Lafayette, IN, USA). Brain slices were kept at ~33˚C in oxygenated ACSF for ~15 min and then allowed to cool at room temperature for at least one hour before we started the recordings.

Electrophysiology

Experiments were carried out with ACSF heated to 32-34°C with a resistive inline heater (Scientifica Ltd), with temperature recorded and verified offline. If outside this range, recordings were truncated or not used. Patch pipettes with a resistance of 4-6 MΩ were pulled using a P-1000 electrode puller (Sutter Instruments, Novato, CA, USA) from medium-wall capillaries. Pipettes were filled with K-gluconate internal solution (containing in mM: KCl, 5: K-Gluconate, 115; K-HEPES, 10; MgATP, 4; NaGTP, 0.3; Na-Phosphocreatine, 10; and 0.1% w/v Biocytin, adjusted with KOH to pH 7.2-7.4 and sucrose to osmolality of 310 mOsm (Abrahamsson et al., 2017). 40 µM and 80 µM Alexa Fluor 594 or Alexa Fluor 488 dyes, respectively, was supplemented to internal solution to permit post-hoc analysis of cell morphology with two-photon laser-scanning microscopy (Blackman et al., 2014; Lalanne et al., 2016). Neurons were patched at 400x magnification guided by infrared video Dodt contrast (built in-house with Thorlabs equipment) on a customized microscope (SliceScope, Scientifica Ltd UK). Primary visual cortex (V1) was distinguished from surrounding V2 based on the presence of cortical layer 4. Pyramidal cells (PCs) in L5 of V1 were targeted based upon their prominent apical dendrite and large triangular somata. Morphometry and cell identity were verified using 2-photon microscopy of Alexa 594/488 fluorescence. Whole-cell recordings were carried out using BVC-700A amplifiers (Dagan Corporation, Minneapolis, MN). Recordings in current-clamp mode were acquired at 10 kHz with PCI-6229 boards (National Instruments, Austin, TX) using our in-house MultiPatch software (Watanabe et al., 2023) (https://github.com/pj-sjostrom/MultiPatch) running in Igor Pro 7 to 9 (WaveMetrics Inc., Lake Oswego, OR).

Since the rate of connectivity in rodent primary visual cortex among L5 PCs is only 10-15% (Sjöström et al., 2001; Song et al., 2005), quadruple whole-cell recording was employed to enable the simultaneous testing of 12 PC→PC connections (Abrahamsson et al., 2016). GΩ seals were first formed on all four cells followed by rapid successive break-through, to avoid plasticity washout (Lalanne et al., 2016). To identify monosynaptic PC→PC connections in current-clamp mode, five spikes were evoked in each cell at 30 Hz by 5-ms-long current injections of 1.3-nA

magnitude, repeated every 20 seconds, and averaged across 10 repetitions. A 250-ms-long test pulse of -25 pA was used to measure input and series resistance. Spike trains in different cells were separated from one another by 700 ms to prevent accidental induction of long-term plasticity (Abrahamsson et al., 2017; Lalanne et al., 2016; Sjöström et al., 2003). If no PC→PC connections were detected, all four recordings were stopped, and another set of four cells were patched with new pipettes, either nearby or in a new acute slice.

STDP experiments

We induced tLTD by evoking postsynaptic firing 25 ms before presynaptic firing, either at 20 Hz or at 1 Hz (Sjöström et al., 2003), which we refer to as HF and LF tLTD, respectively, throughout the present study. tLTP was induced by evoking presynaptic firing 10 ms before postsynaptic cell firing at 50 Hz (Sjöström et al., 2001).

To ensure good signal-to-noise ratio, we only used PC→PC connections >0.3 mV, like before (Sjöström et al., 2007). During baseline periods, presynaptic cells were typically spiked five times at 30 Hz every 20 seconds, resulting in trains of EPSPs, which we denote $EPSP_1$ through $EPSP_5$. We assessed plasticity by calculating the ratio of $EPSP_1$ amplitude averaged across post-induction and pre-induction periods, as indicated in figures. Synaptic stability was assessed using a *t*-test of Pearson's R for $EPSP_1$ across the pre-induction baseline period, with $p < 0.05$ implying instability. Recordings with unstable baseline, > 30% change in input resistance, or > 8 mV change in resting membrane potential were truncated or discarded, like before (Sjöström et al., 2001, 2003, 2007).

To determine the locus of neocortical STDP expression, analysis of the coefficient of variation (CV) of $EPSP_1$ was carried out as previously described (Brock et al., 2020). Briefly, the locus of plasticity expression was determined by the angle φ between the diagonal and the CV endpoint. Presynaptic expression was indicated by $\varphi > 0$ at the $p < 0.05$ level as determined using a standard one-sample *t*-test, whereas postsynaptic expression was similarly indicated by $\varphi < 0$, whereas no significance would suggest a mixed pre- and postsynaptic locus of expression (Sjöström et al., 2007). CV analysis was only carried out with experiments showing at least 5% plasticity, meaning EPSP after/before > 105% for tLTP and EPSP after/before < 95% for tLTD.

Paired-pulse ratio (PPR) was calculated as $(EPSP_2 - EPSP_1)/EPSP_1$, taken from averages before and after tLTD induction. The change in PPR was calculated as $\Delta PPR = PPR_{after} -$

PPR_{before}. A change in PPR after tLTD induction suggested altered probability of release, i.e., a presynaptic locus of expression (Sjöström et al., 2003).

Pharmacology

MK-801 maleate (Hello Bio) and 7-CK (Alomone labs) were washed in at 2 mM and 100 µM, respectively (Nabavi et al., 2013; Sjöström et al., 2003), and slices were incubated for at least 30 minutes before the start of recordings. In JNK2 blockade experiments, slices were incubated in ACSF containing 4 µM SP600125 (Sigma-Aldrich) (Bennett et al., 2001) for at least 2 hours before the start of recordings. This concentration is specific for JNK2 over JNK1 and JNK3 (Abrahamsson et al., 2017; Nisticò et al., 2015).

The peptide used to selectively disrupt JNK2/STX1a interaction was synthesized by Université de Sherbrooke and corresponds to 12 residues (IEQSIEQEEGLNRS) that are part of the N-terminal amino acid sequence of STX1a interacting with JNK2 (Marcelli et al., 2019). Patch pipettes were loaded with 10 µM of the peptide. Neurons were patched for at least 30 minutes before tLTD induction.

Statistics

Unless stated otherwise, results are reported as the mean ± standard error of the mean, with n indicating number of connections. Boxplots show medians and quartiles, with whiskers denoting extremes. Significance levels are denoted using asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Pairwise comparisons were carried out using a two-tailed Student's *t*-test for equal means, unless otherwise indicated. If the equality of variances *F* test gave $p < 0.05$, we employed the unequal variances *t*-test. Wilcoxon-Mann-Whitney's non-parametric test was used in parallel, with similar outcome to the *t*-test. Multiple comparisons were carried out using one-way ANOVA with Bonferroni's post-hoc correction. Pairwise comparisons were only made if ANOVA permitted it at $p < 0.05$ level. Based on the outcome of Bartlett's test, we used homo- or heteroscedastic (Welch) ANOVA. Statistical tests were performed in Igor Pro and Prism 7.0 (GraphPad Software).

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Conflict of Interest Statement:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contribution

AT, SR, JAB, and HH-WW carried out experiments. AT, SR, and JAB performed analysis. PJS wrote custom software. AT and PJS wrote the manuscript with input from HH-WW and SR.

Data Availability Statement

The raw data supporting the conclusions of this manuscript is available from the authors.

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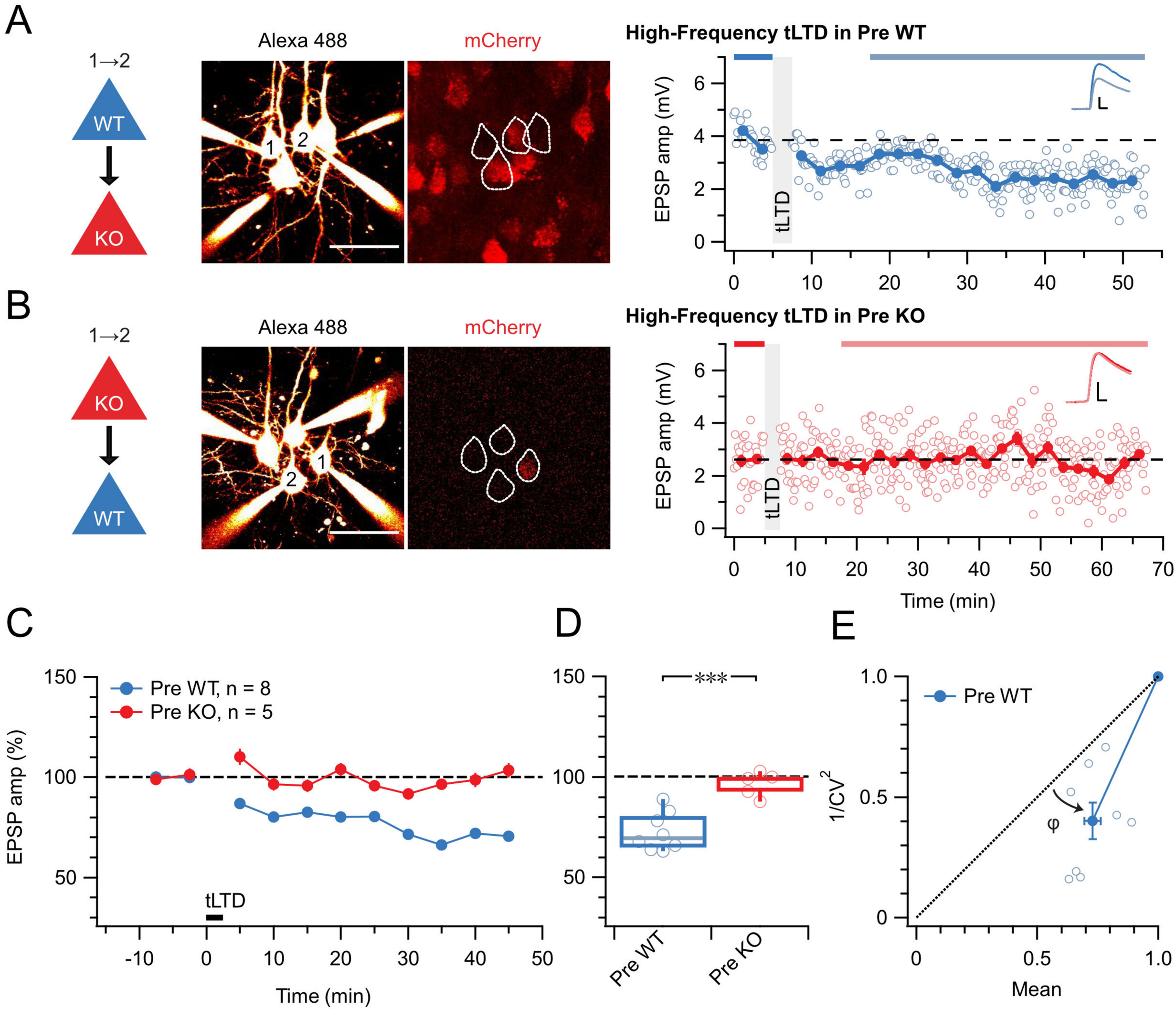
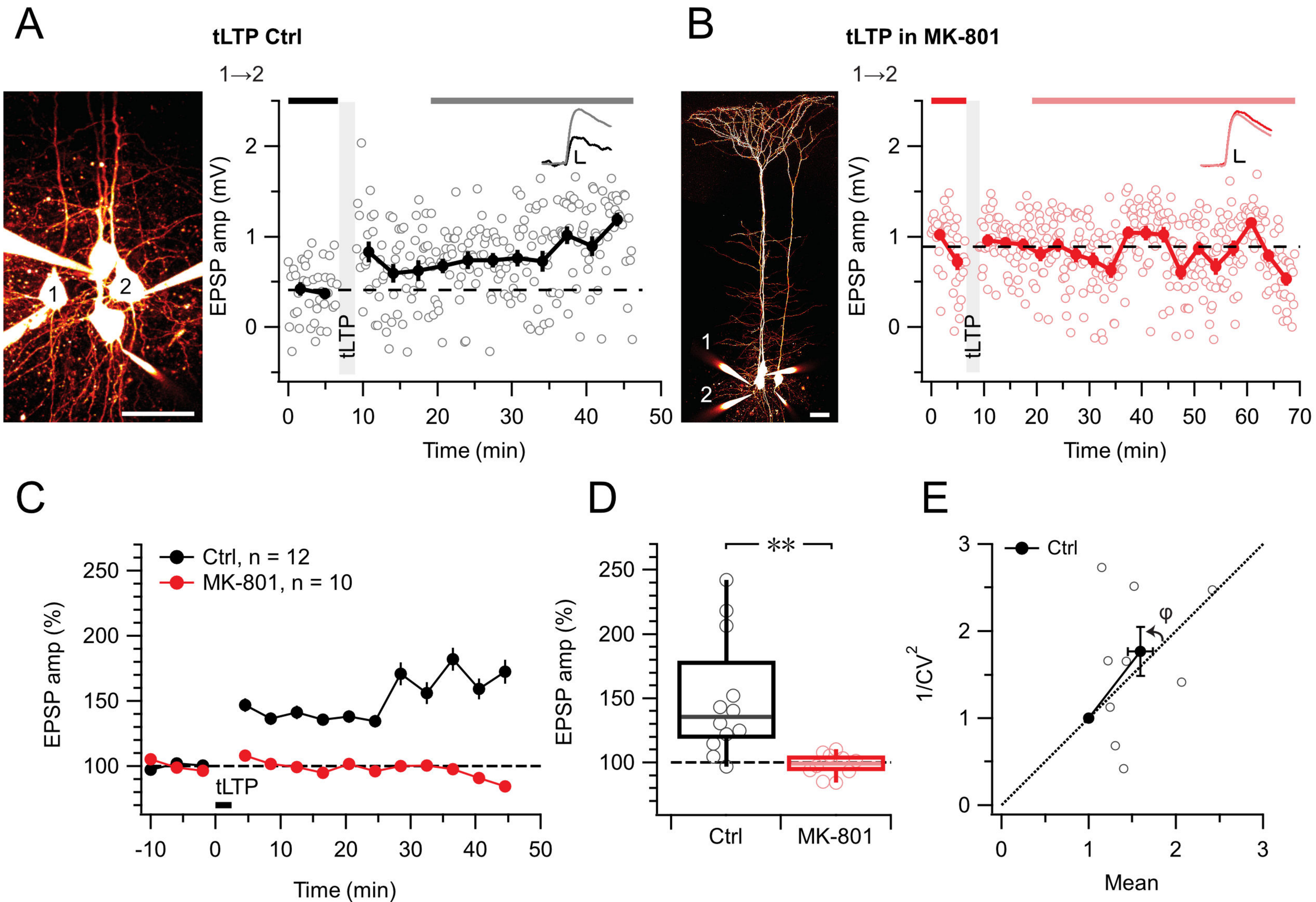
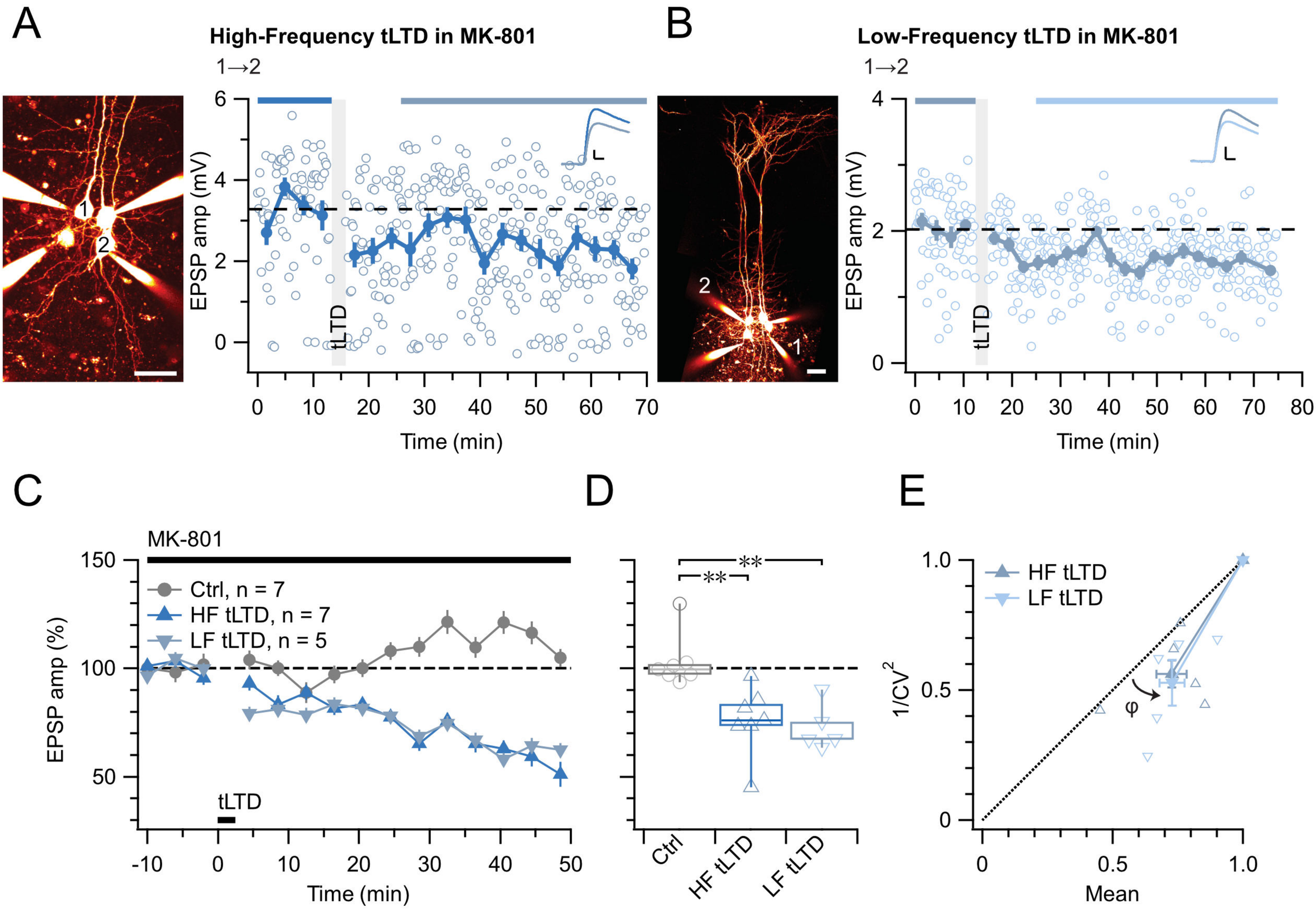
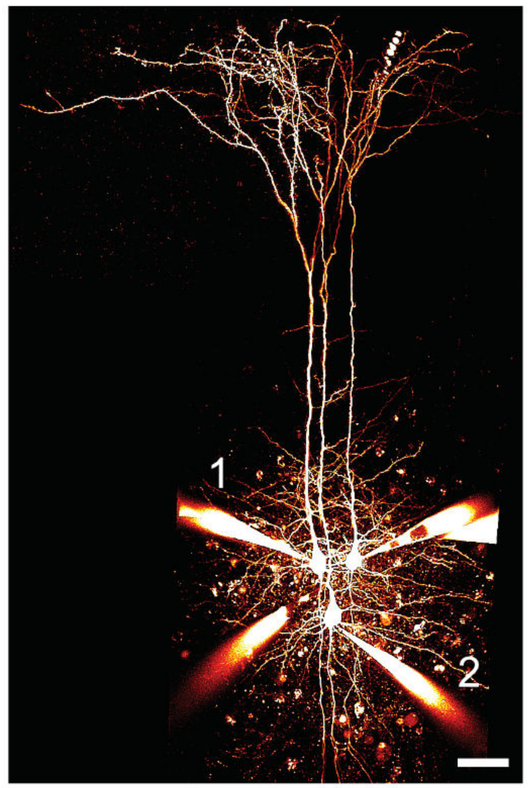
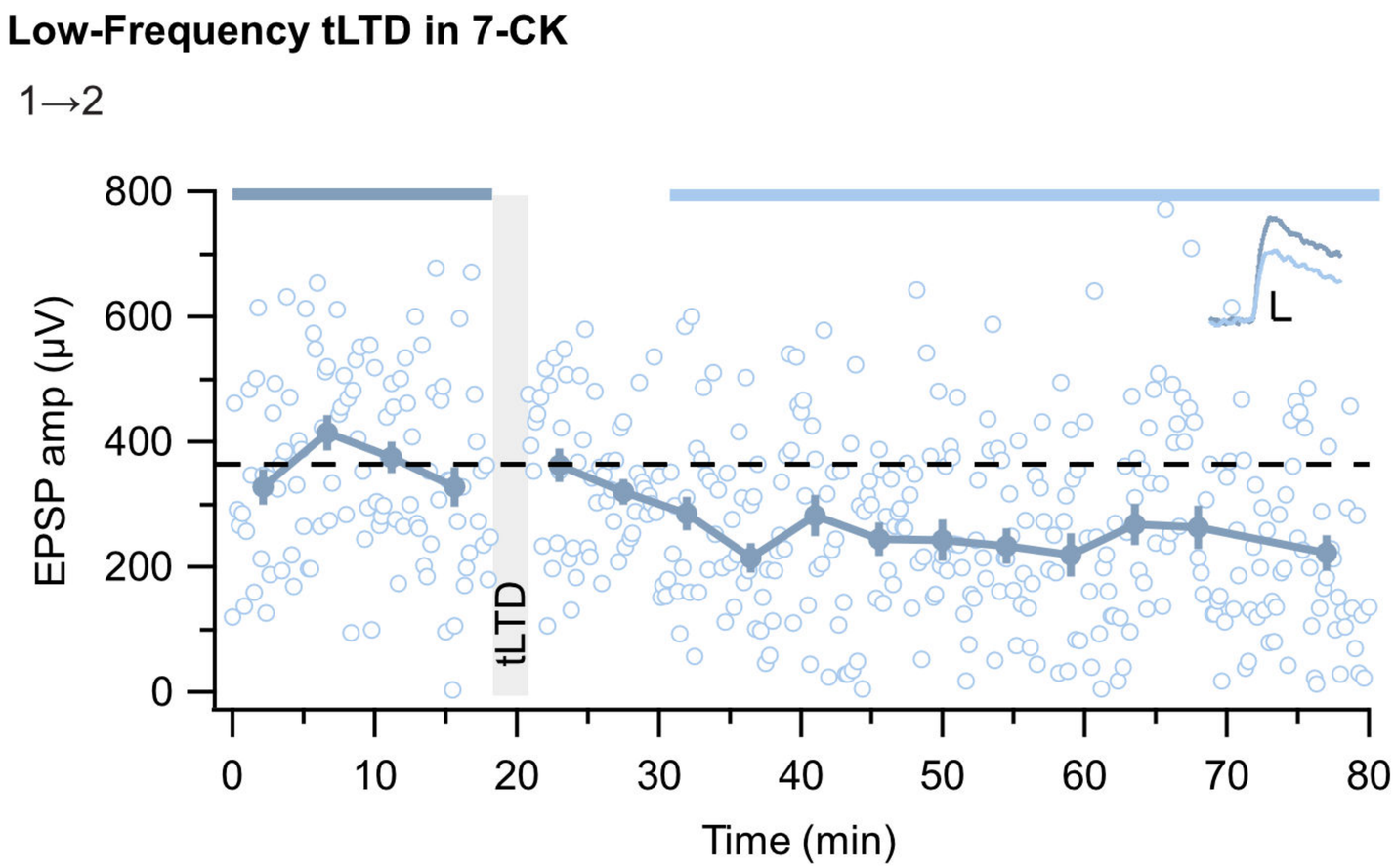

A
1→2
WT
KO
Alexa 488
mCherry
1
2
High-Frequency tLTD in Pre WT
EPSP amp (mV)
tLTD
L
Time (min)
B
1→2
KO
WT
Alexa 488
mCherry
1
2
High-Frequency tLTD in Pre KO
EPSP amp (mV)
tLTD
L
Time (min)
C
Pre WT, n = 8
Pre KO, n = 5
EPSP amp (%)
tLTD
Time (min)
D
***
Pre WT
Pre KO
E
Pre WT
1/CV²
φ
Mean


A
tLTP Ctrl
1→2
EPSP amp (mV)
tLTP
Time (min)
B
tLTP in MK-801
1→2
EPSP amp (mV)
tLTP
Time (min)
C
Ctrl, n = 12
MK-801, n = 10
EPSP amp (%)
tLTP
Time (min)
D
**
EPSP amp (%)
Ctrl
MK-801
E
Ctrl
φ
1/CV²
Mean

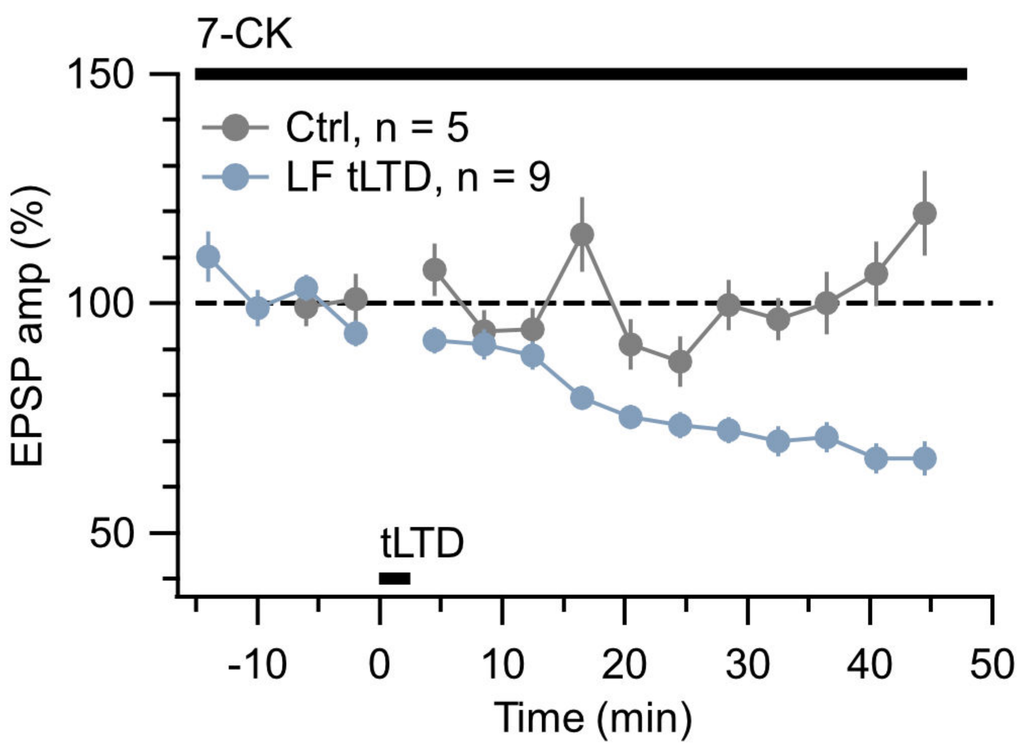

A
High-Frequency tLTD in MK-801
1→2
EPSP amp (mV)
tLTD
Time (min)
B
Low-Frequency tLTD in MK-801
1→2
EPSP amp (mV)
tLTD
Time (min)
C
MK-801
Ctrl, n = 7
HF tLTD, n = 7
LF tLTD, n = 5
EPSP amp (%)
tLTD
Time (min)
D
Ctrl
HF tLTD
LF tLTD
E
HF tLTD
LF tLTD
1/CV²
Mean
φ

A

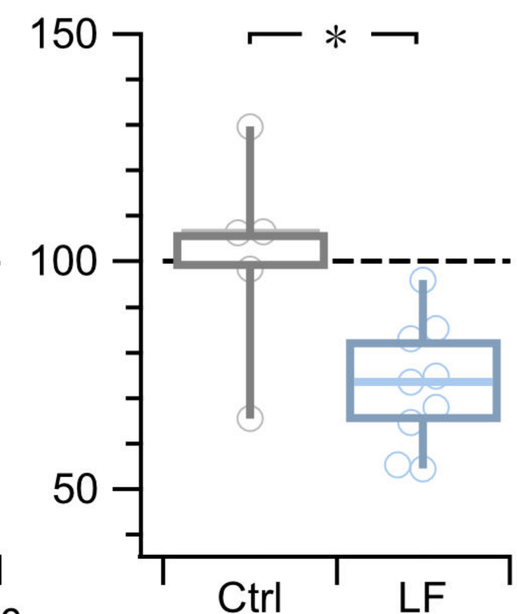

1
2


Low-Frequency tLTD in 7-CK
1→2
EPSP amp (µV)
800
600
400
200
0
tLTD
L
0
10
20
30
40
50
60
70
80
Time (min)

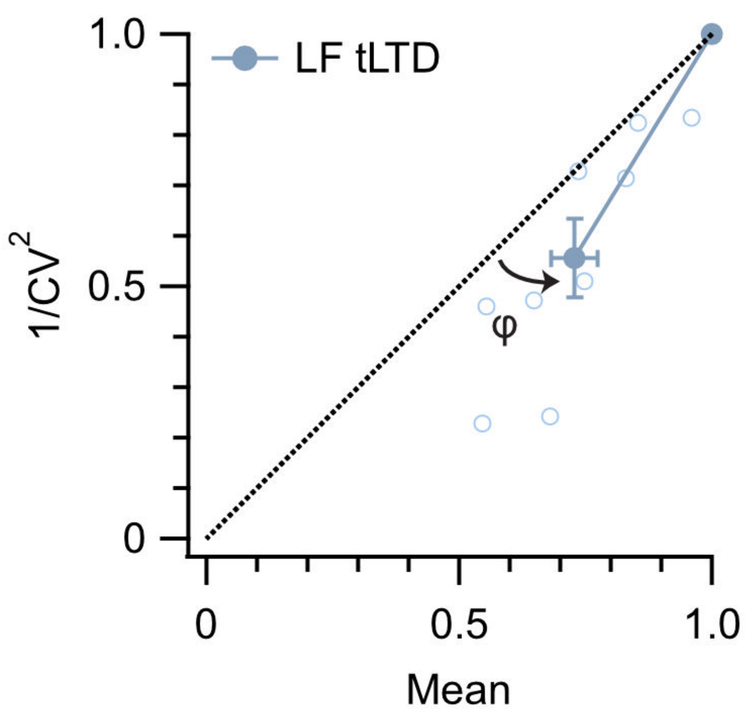
B

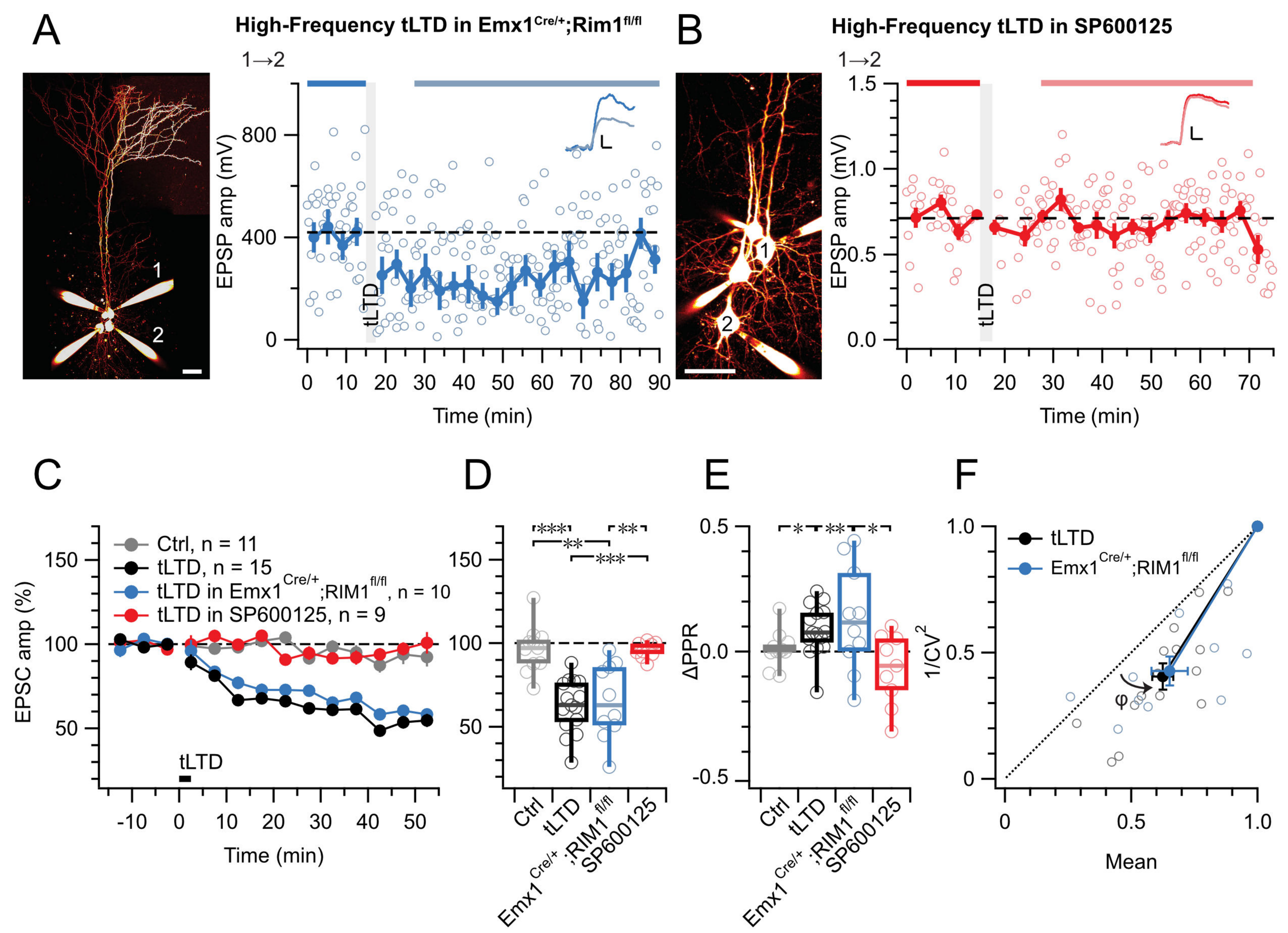

7-CK
Ctrl, n = 5
LF tLTD, n = 9
EPSP amp (%)
150
100
50
tLTD
-10
0
10
20
30
40
50
Time (min)

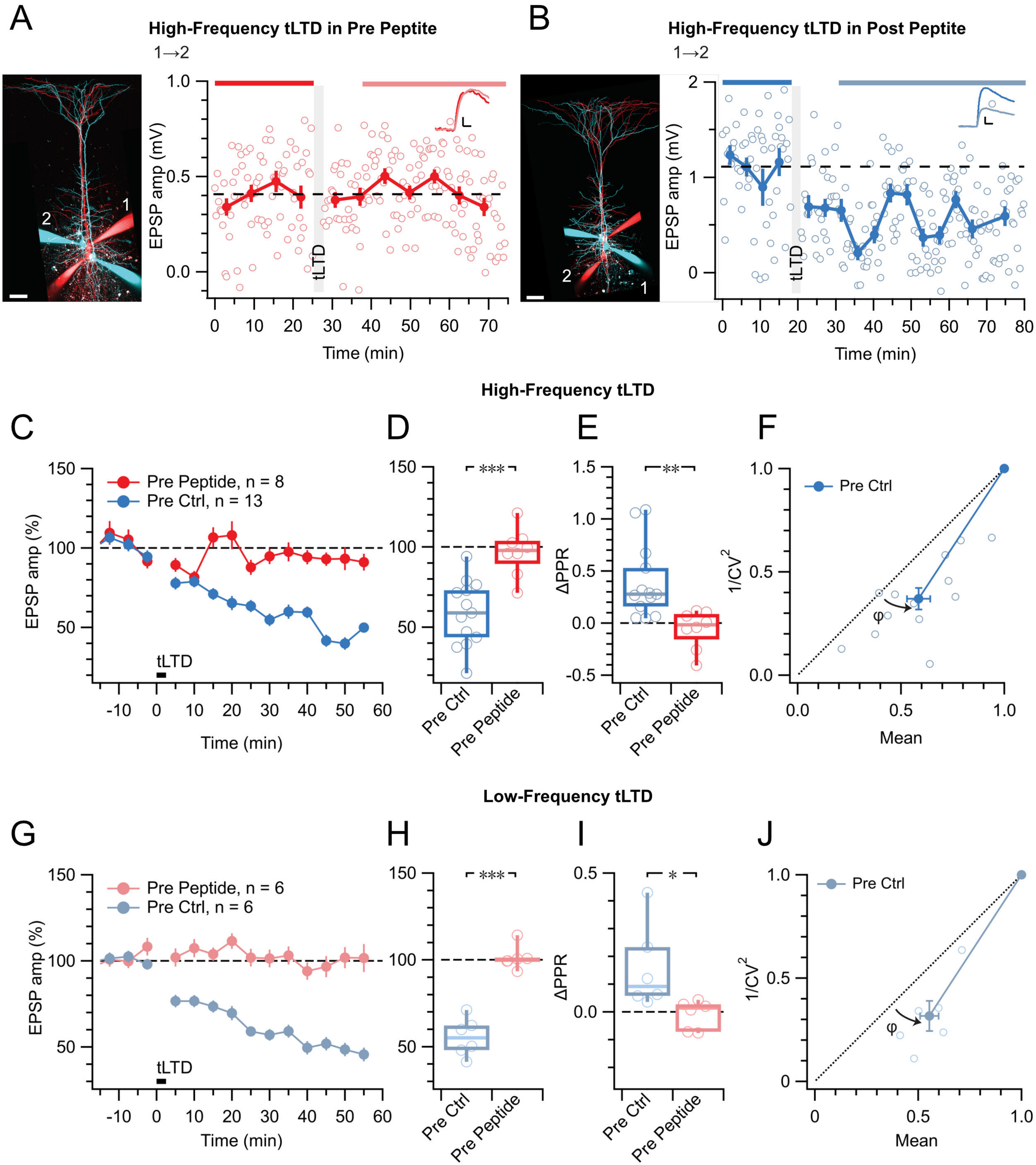
C


150
100
50
*
Ctrl
LF

D


LF tLTD
1/CV²
1.0
0.5
0
φ
0
0.5
1.0
Mean


A
High-Frequency tLTD in Emx1Cre/+;Rim1fl/fl
1→2
EPSP amp (mV)
800
400
0
tLTD
L
1
2
0 10 20 30 40 50 60 70 80 90
Time (min)
B
High-Frequency tLTD in SP600125
1→2
1.5
1.0
0.5
0.0
EPSP amp (mV)
tLTD
L
1
2
0 10 20 30 40 50 60 70
Time (min)
C
Ctrl, n = 11
tLTD, n = 15
tLTD in Emx1Cre/+;RIM1fl/fl, n = 10
tLTD in SP600125, n = 9
EPSC amp (%)
150
100
50
tLTD
-10 0 10 20 30 40 50
Time (min)
D
***
**
**
***
150
100
50
Ctrl
tLTD
Emx1Cre/+;RIM1fl/fl
SP600125
E
0.5
0.0
-0.5
ΔPPR
*
**
*
Ctrl
tLTD
Emx1Cre/+;RIM1fl/fl
SP600125
F
1.0
0.5
0
1/CV2
tLTD
Emx1Cre/+;RIM1fl/fl
φ
0 0.5 1.0
Mean


A
High-Frequency tLTD in Pre Peptite
1→2
EPSP amp (mV)
tLTD
Time (min)
B
High-Frequency tLTD in Post Peptite
1→2
EPSP amp (mV)
tLTD
Time (min)
High-Frequency tLTD
C
Pre Peptide, n = 8
Pre Ctrl, n = 13
EPSP amp (%)
tLTD
Time (min)
D
***
Pre Ctrl
Pre Peptide
E
**
ΔPPR
Pre Ctrl
Pre Peptide
F
Pre Ctrl
1/CV²
Mean
φ
Low-Frequency tLTD
G
Pre Peptide, n = 6
Pre Ctrl, n = 6
EPSP amp (%)
tLTD
Time (min)
H
***
Pre Ctrl
Pre Peptide
I
*
ΔPPR
Pre Ctrl
Pre Peptide
J
Pre Ctrl
1/CV²
Mean
φ