Title: Postsynaptic induction and presynaptic expression of long-term potentiation at excitatory synapses on layer 2/3 VIP interneurons in the somatosensory cortex

Running title: LTP at VIP-INs in the neocortex

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Abstract

Aims: The complex function of the neocortex depends on neuronal circuits composed of highly interconnected excitatory (glutamatergic) neurons and diverse types of inhibitory (GABAergic) interneurons. Synaptic transmission between specific connection motifs undergoes plastic changes during learning process, however, exact mechanisms underlying synaptic plasticity are still under intense investigation. Long-term potentiation (LTP) of synaptic transmission is a widely used cellular model of synaptic plasticity occurring during learning. Here, we focused on studying LTP at excitatory synapses on layer (L) 2/3 vasoactive intestine polypeptide-expressing interneurons (VIP-INs) in the mouse somatosensory (barrel) cortex.

Methods: Acute brain slices were prepared from transgenic mice with fluorescently labeled VIP-INs. LTP was induced by a pairing protocol of postsynaptic depolarization with extracellular stimulation.

Results: The pairing protocol evoked LTP in L2/3 VIP-INs in control condition, however, pharmacological blocking GABAaR inhibition enhanced LTP. Looking for mechanisms of LTP induction, we found that LTP at excitatory connections to VIP-INs is dependent on metabotropic glutamate receptor type 1 (mGluR-1) and L-type voltage-gated calcium channels (L-type VGCC) but not on NMDARs nor mGluR-5. Next, we showed that mGluR-1 acts through G-coupled signaling, Src-family pathway, independently of transient receptor potential channels (TRPC). Analyses of paired-pulse ratio (PPR) and coefficient of variation (CV) indicated a presynaptic locus of LTP expression. Presynaptic expression of LTP in VIP-INs relies on retrograde signaling through endocannabinoids (eCBs) but not on brain-derived neurotrophic factor (BDNF).

Conclusions: We dissected mechanisms of LTP induction and expression at excitatory inputs to L2/3 VIP-INs in the mouse barrel cortex. LTP at excitatory synapses on VIP-INs might serve as a positive feedback for enhanced VIP-IN-mediated inhibition of SST-INs, leading to disinhibition of excitatory neurons from SST-IN inhibition during learning process.

Highlights:

- GABAaR blockage enhances LTP at excitatory inputs to VIP-INs
- LTP in VIP-INs is NMDAR-independent
- Induction of LTP in VIP-INs is mediated by mGluR-1 but not mGluR-5
- mGluR-1 acts through Src-family signaling pathway, independently of TRPC
- L-type VGCC provide calcium influx necessary for LTP induction
- eCBs but not BDNF act as retrograde signaling in LTP in VIP-INs

Keywords: Long-term potentiation, VIP interneurons, NMDA receptor, mGlu receptor, inhibition, synaptic plasticity, learning and memory, barrel cortex, neocortex

Abbreviations

ACSF – artificial cerebrospinal fluid, BDNF – brain-derived neurotrophic factor, CBR-1 – cannabinoid receptor 1, CP-AMPAR – calcium permeable-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, CV – coefficient of variation, eCBs – endocannabinoids, EPSCs – excitatory postsynaptic currents, GABAaR – γ-aminobutyric acid type A receptor, L-type VGCC – long-lasting voltage-gated calcium channel, LTD – long-term depression, LTP – long-term potentiation, mGluR – metabotropic glutamate receptor, NMDAR – N-methyl-D-aspartate receptor, PKC – protein kinase C, PPR – paired pulse ratio, TrkB – tropomyosin receptor kinase B, TRPC – transient receptor potential channel, VGCC – voltage-gated calcium channel, VIP-INs – vasoactive intestinal polypeptide-expressing interneurons

Introduction

It has been believed that synaptic plasticity underlies learning process and memory formation (Martin *et al.*, 2000). Several lines of evidence indicate that mechanisms underlying synaptic plasticity are cell type-, synapse- and brain region-specific (Maffei *et al.*, 2004; Topolnik *et al.*, 2006; Banerjee *et al.*, 2009; Eng *et al.*, 2016; Booker *et al.*, 2018; Williams & Holtmaat, 2019; Chistiakova *et al.*, 2019; Patton *et al.*, 2024). Synaptic plasticity shapes neuronal circuitry, modulating both excitatory (glutamatergic) neurons and inhibitory (GABAergic) interneurons (Wang & Maffei, 2014; Williams & Holtmaat, 2019; Field *et al.*, 2020). Neocortical GABAergic interneurons, even though they represent merely 20% of the entire neuronal population, are immensely divergent group of cells. Vasoactive intestinal polypeptide-expressing interneurons (VIP-INs) constitute around 13% of all neocortical interneurons (Prönneke *et al.*, 2015), but are highly abundant in L2/3 of the barrel cortex (Jiang *et al.*, 2015; Prönneke *et al.*, 2015). Most of L2/3 VIP-INs are characterized by bipolar morphology with dendritic arborizations in L1 and also across all deeper cortical layers (Jiang *et al.*, 2015, 2023; Prönneke *et al.*, 2015; Georgiou *et al.*, 2022). For this reason, it has been suggested that L2/3 VIP-INs might receive excitatory inputs from local excitatory neurons in all cortical layers as well as from long-range projecting neurons (Lee *et al.*, 2013; Prönneke *et al.*, 2015; Audette *et al.*, 2018; Williams & Holtmaat, 2019; Naskar *et al.*, 2021; Georgiou *et al.*, 2022; Jiang *et al.*, 2023). L2/3 VIP-INs provide disinhibition of glutamatergic neurons from other interneurons (Chéreau *et al.*, 2022), especially from somatostatin interneurons (SST-INs) (Lee *et al.*, 2013; Jiang *et al.*, 2015; Walker *et al.*, 2016). VIP-INs are one of the least studied class of interneurons in terms of their plasticity, only few research groups have reported plasticity in VIP-INs (Canto-Bustos *et al.*, 2022; Kanigowski & Urban-Ciecko, 2024, 2025; McFarlan *et al.*, 2024*b*, 2024*a*), albeit disinhibition mediated by VIP-INs is crucial for plasticity during learning process (Lee *et al.*, 2013; Fu *et al.*, 2015; Williams & Holtmaat, 2019; Canto-Bustos *et al.*, 2022).

Long-term potentiation (LTP) of synaptic transmission is a widely used cellular model of synaptic plasticity underlying learning and memory process (Nicoll, 2017). LTP is a long-lasting strengthening of synapses with various underlying mechanisms of induction and expression (Citri & Malenka, 2008). LTP induction has been observed to be predominantly NMDAR-dependent (Jia *et al.*, 1998; Citri & Malenka, 2008; Banerjee *et al.*, 2009; Hasan *et al.*, 2013; Cui *et al.*, 2016; Williams & Holtmaat, 2019; Gómez *et al.*, 2021; Hirai *et al.*, 2022; Patton *et al.*, 2024). Additionally there have been other factors reported that can influence LTP, such as metabotropic glutamate receptors (mGluRs), calcium-permeable AMPARs (CP-AMPARs), or cholinergic receptors

(Topolnik *et al.*, 2005, 2006; Alkadhi, 2021; McFarlan *et al.*, 2023). mGluRs belong to the G-protein coupled receptor family. Class I consists of mGluR-1 and -5 subtypes that may trigger different signaling pathways leading to LTP or long-term depression (LTD) (Perez *et al.*, 2001; Benquet *et al.*, 2002; Gubellini *et al.*, 2003; Topolnik *et al.*, 2005, 2006; Nahir *et al.*, 2010; Kubota *et al.*, 2014; Eng *et al.*, 2016). Expression of LTP depends on pre- or postsynaptic mechanisms (Nicoll, 2003; Citri & Malenka, 2008; Yang & Calakos, 2013). Postsynaptic expression involves potentiation of AMPAR activity through receptor phosphorylation or insertion of new receptors to the postsynaptic membrane (Citri & Malenka, 2008; De León-López *et al.*, 2025), whereas presynaptic expression of LTP relies on an increase of neurotransmitter release probability (Bender *et al.*, 2009; Yang & Calakos, 2013). It has been found that in the motor cortex, LTP at excitatory synapses on L2/3 VIP-INs is expressed postsynaptically but induction mechanisms has not been established (McFarlan *et al.*, 2024*b*).

Here, we described LTP at excitatory synapses on L2/3 VIP-INs in the mouse somatosensory (barrel) cortex. We identified receptors and channels crucial for LTP induction and propose a retrograde signaling factor necessary for LTP expression at these synapses. We found that in the somatosensory cortex LTP induction depends on mGluR-1 and long-lasting voltage-gated calcium channel (L-type VGCC) but not on NMDARs, whereas endocannabinoids (eCBs) act as retrograde messenger for presynaptic expression of LTP through the activation of cannabinoid receptor 1 (CBR-1).

Experimental procedures

Ethics statement

All experiments were performed in compliance with the European Community Council Directive (86/609/EEC) and with the Act on the Protection of Animals Used for Scientific or Educational Purposes in Poland (Act of 15 January 2015, changed 17 November 2021; directive 2010/63/EU) considering transgenic mice employment in research. Animal handling and procedures were conducted in accordance with the Institutional Animal Care and Use at the Nencki Institute guidelines.

Animals

Mice strains were purchased from the Jackson Laboratory. Heterozygous VIP-Cre::Ai14 offspring of homozygous VIP-Cre females bred with homozygous Ai14 males (stock #010908 and #007908

respectively) were used (both sexes, at the age of P21-P28). Mice were weaned at P21 and group housed (according to sex) with unlimited food and water availability, in controlled condition of 12-hour day to night cycle, stable temperature and humidity.

Acute brain slices preparation

For brain extraction, animals were humanely killed by decapitation following deep anesthesia with isoflurane. Slices were then obtained in a paracoronal plane to preserve the barrel field architecture (Urban-Ciecko *et al.*, 2018; Kanigowski *et al.*, 2023; Bogaj *et al.*, 2023). Slices were sectioned to 350 µm with Leica Vibratome, in cold artificial cerebrospinal fluid (ACSF). For recovery and recording, slices were maintained in warm ACSF, heated up to 31 °C. The solution contained (mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 11 glucose and was bubbled with 95%/5% O_2/CO_2 (Urban-Ciecko *et al.*, 2018; Bogaj & Urban-Ciecko, 2025).

Electrophysiology

Neurons were visualized under water-immersion lens with 40x magnification (Axio Examiner A1, Zeiss). In vitro whole-cell patch-clamp technique was performed from fluorescently-labeled VIP-INs in L2/3 of the mouse somatosensory (barrel) cortex. Recordings were acquired with borosilicate glass electrodes (4-7 MΩ), filled with internal solution composed of (mM): 130 cesium gluconate, 10 HEPES, 0.5 EGTA, 8 NaCl, 10 TEA-Cl, 4 Mg-ATP, and 0.3 Na-GTP, adjusted to pH 7.3 and osmolarity 290 mOsm (Kanigowski *et al.*, 2023).

To stimulate excitatory inputs to VIP-INs, bipolar electrode made from theta glass filled with ACSF, was placed in L2/3 below recorded cell (Fig. 1A). Membrane potential of a neuron was held at -65 mV in voltage-clamp mode to acquire evoked excitatory postsynaptic currents (EPSCs). EPSCs were recorded for a stable 5-10 min baseline at the frequency of 0.1 Hz. The intensity of stimulation (Isolator ISO-200, CircleLabs) was set individually for each neuron, to produce minimal stable response to the stimulus lasting 0.2 ms. The EPSC amplitude was analyzed within the area of 3-5 ms after the stimulus artifact. LTP was induced by postsynaptic depolarization to 0 mV paired with the extracellular stimulus, 55-60 consecutive times at the same frequency as for baseline stimulation (Feldman, 2000). Evoked EPSCs were then recorded as in baseline protocol at -65 mV for at least 30 min after the pairing protocol. LTP was assessed 25-30 min after the end of the LTP pairing. For paired-pulse stimulation, 2 pulses were delivered in 50 ms intervals with the frequency of 0.1 Hz (5-10 repetitions after baseline and 30 min after the LTP protocol).

Access resistance (R_a) was monitored throughout recordings. Neurons with a change in R_a over 30% or exhibiting unstable baseline were excluded from further analysis.

Data was acquired using Multiclamp 700B amplifier and digitized with Digidata 1550B (Molecular Devices). Data was filtered at 3 kHz, sampled at 20 kHz and collected by pClamp 10 (Molecular Devices).

In vitro pharmacology

The following drugs were bath applied throughout the recordings: GABAaR antagonist – Bicuculline methiodide (Sigma-Aldrich), 5 μM; NMDAR antagonist – APV, 50 μM; mGluR-5 antagonist – MTEP, 10 μM; mGluR-1 antagonist – LY367385, 50 μM; inhibitor of Src-family tyrosine kinases – PP2, 25 μM; TRPC antagonist – SKF96365, 10 μM; L-type VGCC antagonist – Nifedipine (Sigma-Aldrich), 10 μM; TrkB receptor antagonist – ANA-12, 10 μM; and CBR-1 antagonist – AM-251 (HelloBio), 10 μM. GDPβS (Jena Bioscience), 1mM was applied in the internal solution replacing GTP. Unless indicated otherwise, all drugs were acquired from Tocris Bioscience. Slices were washed with drug solution for at least 10 min prior to data acquisition.

Data analysis

Dataset was analyzed with Clampfit software (Molecular Devices) and in Python environment. The EPSC amplitude was calculated within 3-5 ms after stimulation artifact and normalized to the mean baseline events. Paired pulse ratio (PPR) was assessed as $EPSC_2/EPSC_1$ for baseline and 30 min post-LTP protocol. Coefficient of variation (CV) was analyzed as standard deviation of amplitude divided by averaged amplitude of 30 consecutive sweeps. Then inverted squared CV ($1/CV^2$) was calculated, and normalized $1/CV^2$ against normalized mean amplitude was plotted to assess pre- or postsynaptic locus of LTP expression (Brock *et al.*, 2020). If averaged dataset lays on the unity line (y = x), expression of LTP occurred due to mixed pre- and postsynaptic changes, whereas mean datapoint below or above y = x line indicates postsynaptic or presynaptic locus of plasticity expression, respectively.

Statistical analysis

Data is presented as mean ± standard error of mean (SEM). Normal distribution was checked using Shapiro-Wilk test. To assess LTP, one sample t-test was used. For analysis of two groups, paired t-test, Wilcoxon test or independent t-test were used, as noted. Difference of $p < 0.05$ was considered statistically significant. Statistics and data visualization were obtained with Python.

Results

Pharmacological blockage of GABAaRs facilitates LTP at excitatory synapses on L2/3 VIP-INs

To study synaptic plasticity of excitatory synapses on L2/3 VIP-INs in the somatosensory cortex, we performed whole-cell patch-clamp recordings in acute brain slices (Fig. 1A) obtained from transgenic mice with VIP-INs expressing td-Tomato fluorescent protein. LTP was evoked by extracellular electrical stimulation of excitatory terminals paired with membrane depolarization of a patched cell (Feldman, 2000). Stimulating electrode was placed in L2/3 below recorded interneurons (Fig. 1A) to recruit local inputs (Porter *et al.*, 1998).

First, we performed the pairing protocol in control condition with no drugs administered. We managed to trigger LTP; the EPSC amplitude increased by 63 % when measured 30 min after the induction protocol (Fig.1B, normalized amplitude: 1.63 ± 0.13, $p = 0.004$, $n = 7$, one sample t-test). Since blocking GABAaR-mediated inhibition facilitates LTP induction in many brain regions (Grover & Yan, 1999), we then asked whether inhibition mediated via these receptors influences LTP induction at excitatory synapses on L2/3 VIP-INs. Bath application of the GABAaR antagonist (bicuculline, bicc) evoked LTP (Fig. 1B, normalized amplitude: 2.43 ± 0.25, $p = 0.002$, $n = 7$, one sample t-test) and, indeed, significantly enhanced LTP in comparison to no drug condition ($p = 0.022$, independent t-test). For this reason, all the following experiments were performed in the presence of bicuculline in ACSF.

To characterize a locus of LTP expression, we analyzed PPR and $1/CV^2$ (McFarlan *et al.*, 2024*b*). We observed a significant increase in 1st amplitude (Fig. 1C, pooled data, pre 49.44 ± 6.79 pA vs. post 83.95 ± 14.81 pA, $p = 0.028$, $n = 6$, paired t-test), and a reduction in PPR after LTP induction (Fig. 1D, pooled data, pre 1.41 ± 0.34 vs. post 0.94 ± 0.12, $p = 0.031$, $n = 6$, Wilcoxon test), together suggesting an increase of glutamate release probability. Furthermore, $1/CV^2$ analysis showed possible presynaptic locus of LTP expression, since most of the datapoints are scattered above the unity line indicating alterations in the presynaptic site (Fig. 1E).

LTP at excitatory synapses on VIP-INs is NMDAR independent

Next, we aimed to uncover mechanisms of LTP induction. Since the protocol evoking LTP relies on membrane depolarization, we searched for glutamate- and voltage-dependent mechanisms. We rejected the possibility that CP-AMPA receptors mediate LTP, because depolarization to 0 mV leads to channel pore blockage by endogenous polyamines (Bowie & Mayer, 1995; Topolnik *et al.*, 2005). Also, it has been shown that CP-AMPARs do not participate in LTP in VIP-INs in the motor cortex (McFarlan *et al.*, 2024*a*). However, it has been well established that NMDARs are

voltage-sensitive (Nowak *et al.*, 1984; Mayer *et al.*, 1984; Vargas-Caballero & Robinson, 2004), hence we blocked those receptors with APV to assess their putative role in induction of LTP at excitatory synapses on L2/3 VIP-INs. Surprisingly, the NMDAR antagonist failed to prevent LTP induction at these synapses (Fig. 2A, normalized amplitude 1.85 ± 0.17, p = 0.001, n = 10, one sample t-test). Moreover, further analysis showed enhancement of the first amplitude (Fig. 2B, pre 66.35 ± 2.67 pA vs. post 121.24 ± 17.59 pA, p = 0.025, n = 6, paired t-test), diminished PPR (Fig. 2C, pre 1.28 ± 0.08 vs. post 1.09 ± 0.05, p = 0.049, n = 6, paired t-test) and increased $1/CV^2$ (Fig. 2D), hinting towards a presynaptic locus of LTP expression.

Postsynaptic mGluR-1 is responsible for LTP in VIP-INs

After establishing that NMDARs are not involved in LTP induction at excitatory synapses on L2/3 VIP-INs, we searched for another neurotransmitter targets. Metabotropic glutamate receptors (mGluR-1 and mGluR-5) are expressed in cortical neurons (Kerner *et al.*, 1997; Heidinger *et al.*, 2002) and might be responsible for LTP induction (Huemmeke *et al.*, 2002; Topolnik *et al.*, 2006). Blocking mGluR-5 with MTEP did not abolish LTP after the pairing protocol (Fig. 2E, normalized amplitude 1.64 ± 0.21, p = 0.031, n = 7, one sample t-test; Fig. 2F, amplitude pre 51.7 ± 6.94 pA vs. post 78.41 ± 12.44 pA, p = 0.014, n = 7, paired t-test). Changes in PPR (Fig. 2G, pre 0.89 ± 0.15 vs. post 0.64 ± 0.11, p = 0.042, n = 7, paired t-test) and $1/CV^2$ (Fig. 2H) still suggested the presynaptic locus of LTP expression. However, the pairing protocol failed to induce LTP in VIP-INs in the presence of the mGluR-1 antagonist (LY367385) (Fig. 3A, normalized amplitude1.18 ± 0.22, p = 0.257, n = 9, one sample t-test; Fig. 3B, amplitude pre 64.6 ± 8.27 pA vs. post 62.13 ± 9.33 pA, p = 0.700, n = 9, paired t-test). Also, there were no changes in PPR (Fig. 3C, pre 1.10 ± 0.11 vs. post 1.36 ± 0.17, p = 0.185, n = 9, paired t-test) and $1/CV^2$ (Fig. 3D) in this condition.

Since mGluRs are G-protein coupled receptors but can be expressed both pre- and postsynaptically (Falcón-Moya *et al.*, 2020) and astrocyte also can be involved in signaling through mGluRs (Kofuji & Araque, 2021), we asked whether mGluRs-1 modulating LTP in VIP-INs are located postsynaptically. To address this question, we loaded VIP-INs with GDPβS, an inhibitor of G-protein coupled signaling pathway. GDPβS blocked LTP induction indicating the postsynaptic action of mGluRs-1 in this process (Fig. 3E, normalized amplitude 0.83 ± 0.15, p = 0.374, n = 5, one sample t-test; Fig. 3F amplitude pre 90.30 ± 16.93 pA vs. post 87.59 ± 23.99 pA, p = 0.874, n = 5, paired t-test; Fig. 3G PPR pre 1.21 ± 0.20 vs. post 1.01 ± 0.11, p = 0.305, n = 5, paired t-test; Fig. 3H $1/CV^2$).

mGluR-1 can act through two independent mechanisms in order to produce calcium influx: through activation of Src-family kinases or protein kinase C (PKC) (Topolnik *et al.*, 2006; Eng *et al.*, 2016; Sugawara *et al.*, 2017). To reveal the mode of mGluR-1 action, we applied Src tyrosine kinase antagonist (PP2). In this condition, we could not elicit LTP in VIP-INs (Fig. 4A, normalized amplitude 1.04 ± 0.17, p = 0.255, n = 7, one sample t-test; Fig. 4B, amplitude pre 80.01 ± 3.20 pA vs. post 87.41 ± 19.50 pA, p = 0.688, n = 7, Wilcoxon test). There were also no changes observed in PPR analysis (Fig. 4C, pre 1.16 ± 0.09 vs. post 1.10 ± 0.05, p = 0.669, n = 7, paired t-test), nor in $1/CV^2$ (Fig. 4D).

Further, we wanted to know whether Src pathway leads to activation of transient receptor potential channel (TRPC) (Topolnik *et al.*, 2006; Kubota *et al.*, 2014). Blocking TRPC using SKF96365 did not affect LTP induction (Fig. 4E, normalized amplitude 1.65 ± 0.17, p = 0.008, n = 8, one sample t-test). The EPSC amplitude increased nearly 2-fold post pairing protocol (Fig. 4F, pre 62.06 ± 6.98 pA vs. post 105.78 ± 13.37 pA, p = 0.016, n = 8, Wilcoxon test). PPR significantly decreased (Fig. 4G, pre 1.42 ± 0.14 vs. post 1.06 ± 0.18, p = 0.022, n = 8, paired t-test) and $1/CV^2$ analysis (Fig. 4H) lies above the unity line, showing possible presynaptic locus of synaptic changes.

As we found that TRPC does not mediate LTP induction, we aimed to find other potential target for Src kinase. We targeted L-type voltage-gated calcium channels (L-type VGCC) as they can be coupled with mGluRs (Chavis *et al.*, 1996, 1998; Topolnik *et al.*, 2009; Booker *et al.*, 2018) and activity of these channels can be potentiated by tyrosine kinase family (Man *et al.*, 2023). Moreover, the pairing protocol involves membrane depolarization, which presumably activates VGCC. Indeed, the L-type VGCC blocker (nifedipine) abolished LTP induction in VIP-INs (Fig. 5A, normalized amplitude 0.89± 0.06, p = 0.192, n = 5, one sample t-test; Fig. 5B, amplitude pre 107. 58 ± 28.40 pA vs. post 93.54 ± 22.48 pA, p = 0.147, n = 5, paired t-test; Fig. 5C, PPR pre 0.81 ± 0.13 vs. post 0.83 ± 0.04, p = 0.870, n = 5, paired t-test; Fig. 5D $1/CV^2$).

Together, these findings suggest that postsynaptic mGluR-1 but not mGluR-5 is necessary for LTP of excitatory transmission to L2/3 VIP-INs. LTP induction depends on calcium influx through L-type VGCC but not TRPC.

Presynaptic expression of LTP in VIP-INs

Since PPR and $1/CV^2$ analyses (Fig. 1E, 2D,H, 4H) showed putative presynaptic locus of LTP expression, we next aimed to find a signaling factor acting retrogradely (Nicholson & Kullmann, 2014). First, we checked whether brain-derived neurotrophic factor (BDNF) could be a retrograde messenger to trigger presynaptic changes in VIP-IN excitatory inputs, as it has been shown in

CA1 neurons (Thapliyal *et al.*, 2022) and cortical pyramidal neurons (Maglio *et al.*, 2018). To do this, we blocked tropomyosin receptor kinase B (TrkB), a high-affinity receptor for BDNF, before the LTP pairing protocol. The TrkB receptor antagonist (ANA-12) did not prevent LTP induction (Fig. 6A, normalized amplitude 1.97 ± 0.34, $p = 0.047$, $n = 6$, one sample t-test; Fig. 6B, amplitude pre 65.57 ± 13.89 pA vs. post 108.42 ± 15.47 pA, $p = 0.000$, $n = 6$, paired t-test), indicating that BDNF is not necessary for LTP at excitatory inputs to L2/3 VIP-INs. Just like in our previous experiments, the PPR (Fig. 6C, pre 1.42 ± 0.09 vs. post 1.05 ± 0.09, $p = 0.072$, $n = 6$, paired t-test) and $1/CV^2$ analyses (Fig. 6D) revealed the presynaptic locus for LTP expression.

Another putative retrograde signaling factor could be eCBs, which are sensitive to depolarization and calcium influx. eCBs have been established to be an important messenger in plasticity (Maglio *et al.*, 2018). Indeed, administration of the CBR-1 antagonist (AM-251) blocked LTP in cortical VIP-INs (Fig. 6E, normalized amplitude 0.91 ± 0.11, $p = 0.511$, $n = 4$, one sample t-test; Fig. 6F, amplitude pre 71.48 ± 13.14 pA vs. post 76.86 ± 4.04 pA, $n = 3$; Fig. 6G PPR pre 1.02 ± 0.15 vs. post 0.89 ± 0.14, $n = 3$; Fig. 6H), suggesting that CBR-1 is responsible for presynaptic LTP expression.

In conclusion, our results indicate that expression of LTP at excitatory connections to VIP-INs depends on a retrograde signaling mediated by eCBs but not BDNF.

Discussion

Our findings provide insight into the mechanism of the Hebbian long-term plasticity of excitatory synapses on VIP interneurons in L2/3 of the mouse somatosensory cortex. Here, using electrophysiological approach, we described molecular factors essential for LTP induction and expression in these interneurons. We established that LTP at excitatory inputs to VIP-INs is mediated by postsynaptic activation of mGluR-1 and L-type VGCC (Fig. 7). Moreover, our findings uncovered CBR-1 as the presynaptic target of LTP expression at excitatory synapses on VIP-INs.

It has been hypothesized that LTP cannot occur at excitatory inputs to GABAergic neurons (McBain *et al.*, 1999; Ross & Soltesz, 2001), nevertheless later studies have shown that plasticity direction is brain region- and synapse type- specific (Perez *et al.*, 2001; Topolnik *et al.*, 2006; Sarihi *et al.*, 2008; Oren *et al.*, 2009; Banerjee *et al.*, 2009; Chen *et al.*, 2009; Szegedi *et al.*, 2016; Chistiakova *et al.*, 2019; McFarlan *et al.*, 2024*b*). Similarly to research conducted on excitatory inputs to L2/3 VIP-INs in the motor cortex (McFarlan *et al.*, 2024*b*) and on hippocampal CA1 inputs to SST-INs (Vasuta *et al.*, 2015), we were able to induce LTP at excitatory synapses on L2/3 VIP-

INs in the barrel cortex, even with no pharmacological blockade of GABAaRs. It is worthwhile to mention that synaptic plasticity is an interplay of glutamatergic and GABAergic net force (Wang & Maffei, 2014; Field *et al.*, 2020; Agnes & Vogels, 2024). Many studies have shown that across different brain regions, GABAaRs can either block or conversely, facilitate LTP induction (Ruiz *et al.*, 2010; Paille *et al.*, 2013; Davenport *et al.*, 2021). Indeed, pharmacological blockade of GABAaRs enhanced LTP at excitatory synapses on L2/3 VIP-INs in the barrel cortex, inferring that in physiological conditions those receptors diminish LTP at these connections. It has been suggested that blocking GABAaR-mediated inhibition facilitates postsynaptic membrane depolarization and thus enhances activation of NMDARs or voltage-dependent channels during LTP induction (Grover & Yan, 1999). Since in our study, the postsynaptic neurons were artificially depolarized during the LTP protocol, we assume that release of presynaptic neurons from GABAaR inhibition might have essential impact in facilitation of LTP in this case.

Numerous studies have recognized NMDAR as the primary glutamatergic signaling factor of synaptic plasticity in central nervous system, especially in the hippocampus (Citri & Malenka, 2008; Hirai *et al.*, 2022), but also in thalamocortical inputs to L4 (Lu *et al.*, 2001), in excitatory connections to pyramidal neurons located in L2/3 (Banerjee *et al.*, 2009, 2014; Williams & Holtmaat, 2019) and L5 of the somatosensory cortex (Maglio *et al.*, 2018; Gómez *et al.*, 2021) or in motor cortex (Hasan *et al.*, 2013). NMDARs are vastly expressed in VIP-INs (Porter *et al.*, 1998). The pairing protocol applied to induce plasticity in VIP-INs is highly dependent on postsynaptic calcium signaling triggered by membrane depolarization, favouring the NMDAR activation (Feldman, 2000). For that reason, we expected NMDAR to be a key player necessary for LTP induction in L2/3 VIP-INs of the mouse somatosensory cortex. To our surprise, induction of LTP at glutamatergic synapses on VIP-INs was NMDAR-independent similarly to what has been observed in other class of inhibitory interneurons – SST-INs in the hippocampus and neocortex (Perez *et al.*, 2001; Oren *et al.*, 2009; Chen *et al.*, 2009; Nicholson & Kullmann, 2014). In contrast, LTP is NMDAR-dependent at excitatory inputs to hippocampal parvalbumin interneurons (PV-INs) (Cornford *et al.*, 2019) or between cortical pyramidal cells (Banerjee *et al.*, 2009).

Another putative receptors underlying plasticity formation are mGluRs, or ionotropic receptors such as calcium-permeable AMPARs and nicotinic receptors (Alkadhi, 2021; McFarlan *et al.*, 2023). In the present study, we assessed that mGluR-5 does not contribute to LTP at excitatory inputs to L2/3 VIP-INs, unlike glutamatergic inputs to L2/3 pyramidal neurons (Cui *et al.*, 2018). However, mGluR-1 is necessary for LTP induction in L2/3 VIP-INs, as it has been observed for excitatory synapses on SST-INs in hippocampal CA1 oriens/alveus (Vasuta *et al.*, 2015). PV-INs,

in turns, show mostly mGluR-5-dependent plasticity, possibly since there are hardly any mGluR-1 expressed in those neurons (Sarihi *et al.*, 2008; Vasuta *et al.*, 2015). Interestingly, in human cortex mGluR class I has been implicated in LTD induction in pyramidal to pyramidal connections (Kroon *et al.*, 2019), as well as in excitatory inputs to fast-spiking interneurons (putative PV-INs) (Szegedi *et al.*, 2016). Moreover, there are also neurons that require activity of both mGluR-1 and -5 for LTP induction, as it has been observed in corticostriatal synaptic connections (Gubellini *et al.*, 2003).

Metabotropic receptors regulate calcium ion increase in the postsynaptic cell via different signaling pathways (Mannaioni *et al.*, 2001; Topolnik *et al.*, 2006; Eng *et al.*, 2016). It has been previously discovered that mGluR-1 in CA1 can activate, among others, MAPK cascade mediating synaptic plasticity (Topolnik *et al.*, 2006; Eng *et al.*, 2016). Topolnik et al. (Topolnik *et al.*, 2006) showed that hippocampal O/A interneurons require Src/ERK downstream pathway in order to activate MAPK and thus induce LTP. On the contrary, we uncovered that glutamatergic inputs to cortical VIP-INs are dependent solely on Src tyrosine kinase activity, since blockage of this enzyme abolishes LTP completely, whereas in the hippocampus, LTP required complementary activity of ERK (Topolnik *et al.*, 2006). Additionally, Src activation has been established to lead to calcium ions influx through TRPC (Topolnik *et al.*, 2006; Kubota *et al.*, 2014) but our data excluded TRPC involvement in LTP induction in VIP-INs. However, we found that LTP in these interneurons depended on L-type VGCC, suggesting that depolarization during the pairing protocol profoundly activates these channels leading to calcium influx necessary for LTP induction. L-type VGCC- but not NMDA-dependent LTP has previously been observed in many brain areas including the somatosensory cortex, amygdala and CA1 (Grover & Teyler, 1990; Weisskopf *et al.*, 1999; Cui *et al.*, 2018). Nonetheless, it remains obscure whether Src kinase directly interacts with L-type VGCC in VIP-INs. Previous single-channel recordings of hippocampal neurons have revealed that Src can directly boost L-type VGCC activity (Man *et al.*, 2023), whereas studies on neuronal L-type VGCC expressed in HEK293-T showed physical binding of Src and these channels (Chao *et al.*, 2011). In light of this evidence, we may hypothesize that Src kinase binds to and activates L-type VGCC, or more likely the enzyme enhances the activity of these channels which are probably already activated through depolarization of the postsynaptic membrane during the paring protocol. Also, we cannot rule out a possibility of presynaptic L-type VGCCs as well astrocytic channel involvement in the process of LTP induction (Falcón-Moya *et al.*, 2020). However, we established that LTP at excitatory inputs to L2/3 VIP-INs required postsynaptic G-protein activation, presumably triggered by mGluR-1 activation.

Synaptic plasticity may arise from changes in the postsynaptic or presynaptic membrane or be a result of mixed effects – pre- and postsynaptic mechanisms. It has been proven that a direction of plasticity is age-dependent and stimulation location sensitive (Reyes & Sakmann, 1999; Banerjee *et al.*, 2009, 2014; St. Laurent & Kauer, 2019). Here, even though induction of LTP in VIP-INs was postsynaptic, analyses of PPRs and $1/CV^2$ suggest that LTP expression locus laid in the presynaptic site. Corresponding results based on these analyses have been observed in cortical excitatory inputs to SST-INs (Chen *et al.*, 2009; Nicholson & Kullmann, 2014; Chistiakova *et al.*, 2019) and to hippocampal oriens-lacunosum moleculare (O-LM) interneurons (Oren *et al.*, 2009). On the contrary, postsynaptic LTP expression has been found at glutamatergic inputs to L2/3 VIP-INs in the motor cortex (McFarlan *et al.*, 2024*b*). The discrepancies between our results and McFarlan's observation may result from the following factors: diverse brain regions (somatosensory cortex vs. motor cortex), age (P21-28 vs. P21-40), stimulation protocols (pairing postsynaptic depolarization with external stimulation vs. spike timing-dependent plasticity) and the location of stimulation electrodes (below patched cells vs. above), respectively.

We found that LTP in VIP-INs was depended on the activity of CBR-1, suggesting eCBs as retrograde messengers acting on CBR-1 in the presynaptic site, as reported in L5 and 2/3 pyramidal neurons in the barrel cortex (Maglio *et al.*, 2018; Cui *et al.*, 2018). Furthermore, Maglio et al. study shows that the LTP was also dependent on BNDF, in contrast to our findings in which TrkB activation was not crucial for LTP expression. The location of CBR-1 contributing to synaptic plasticity of connections to VIP-INs, remains undiscovered. These receptors could be located both on the presynaptic site or on astrocytes (Navarrete & Araque, 2010). We hypothesize that depolarization during the LTP protocol leads to eCB release from the postsynaptic site, because it has been established that eCBs are released in an activity-dependent manner (Di Marzo *et al.*, 1998). In general, activation of CBR-1 reduces neurotransmitter release leading to LTD (Sjöström *et al.*, 2003; Chevaleyre & Castillo, 2004; Chevaleyre *et al.*, 2006). Nonetheless, CBR-1 has been implicated in LTP in the striatum and neocortex due to an interplay with the transient receptor potential vanilloid-1 (TRPV1) (Cui *et al.*, 2015, 2018). As TRPV1 are highly permeable to calcium (Ross, 2003), they may contribute to LTP by boosting calcium transient in the presynaptic site which in turn, triggers an increase of neurotransmitter release from excitatory terminals on L2/3 VIP-INs. This mechanism remains yet to be experimentally proven, as in general mechanisms of presynaptic expression of LTP are noted but less explored.

What is a consequence of LTP at excitatory connections to L2/3 VIP-INs in the barrel cortex? Enhanced excitatory transmission to VIP-INs might serve as a positive feedback for stronger VIP-

IN-mediated inhibition of SST-INs, leading to disinhibition of excitatory neurons from SST-IN-mediated inhibition during learning process. On the other hand, greater excitation of VIP-INs might be a part of a pathological mechanism leading to network hyperexcitation, because it has been found that optogenetic and genetic silencing of VIP-INs reduces susceptibility to seizures (Khoshkhoo *et al.*, 2017; Rahimi *et al.*, 2023). In this case, the LTP pairing protocol with prolonged VIP-IN depolarization might serve as a model to study mechanisms of pathological conditions.

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Competing interests

Authors declare no conflict of interests.

Author Contributions

Joanna Urban-Ciecko contributed to the study by conception, design and funding acquisition. Material preparation, data collection and analysis were performed by Karolina Bogaj. All authors wrote, read and approved the manuscript.

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Figures

Fig. 1. Inhibition of GABAaRs elevates LTP at excitatory inputs to L2/3 VIP-INs in the somatosensory cortex. A) Picture of acute brain slice with stimulating and recording electrodes in L2/3 of the mouse barrel cortex, and a schematic illustrating the stimulation paradigm – excitatory inputs from pyramidal cells (Pyr – dark gray) are stimulated to evoke EPSCs in fluorescently-labeled VIP-INs (VIP – red). **B)** Time course plot of normalized EPSC before and after the pairing protocol in control condition (No drug: pre pairing – black circles, post pairing – light gray circles) and in bath application of the GABAaR antagonist (bicuculline, Bicc: pre pairing – solid green circles and post pairing – open green circles). Horizontal dashed line with an asterisk indicates statistically significant differences for LTP induction in each experimental condition, vertical line with an asterisk indicates a significant difference between the two recording conditions. Above, exemplary EPSC traces for pre and post LTP induction protocol. **C)** Averaged 1st amplitude for pre and post LTP induction (pooled data for No drug and GABAaR blockage conditions). **D)** Paired pulse ratio for pre and post LTP induction protocol (pooled data as for C). **E)** Normalized $1/CV^2$ plotted against normalized amplitude for both conditions. Mean ± SEM, * $p < 0.05$.

Fig. 2. LTP on L2/3 VIP-INs is NMDAR and mGluR-5 independent. A) Time course plot of normalized EPSC before and after the pairing protocol in the presence of the NMDAR antagonist (APV); pre pairing – black circles, post pairing – blue circles. Above, exemplificatory EPSC traces for pre and post pairing. **B)** Averaged 1st amplitude for pre and post LTP induction protocol with bath applied NMDAR antagonist. **C)** Averaged paired pulse ratio for pre and post LTP induction in APV. **D)** $1/CV^2$ analysis in APV condition. **E)** Same as in A) but for mGluR-5 blockage (MTEP) condition (pre pairing – black circles, post pairing – turquoise circles). **F-H)** Same as B-D) but with bath applied MTEP. Mean ± SEM, * $p < 0.05$.

Fig. 3. Induction of LTP at excitatory synapses on L2/3 VIP-INs is mediated by postsynaptic mGluR-1 through G-protein signaling pathway. A) Normalized EPSC in bath-applied mGluR-1 antagonist (LY367385) condition (pre pairing – black circles, post pairing – red circles). Exemplificatory EPSC traces for pre and post pairing. **B)** Averaged 1st amplitude for pre and post LTP induction protocol in the presence of LY367385. **C)** Averaged paired pulse ratio pre and post LTP induction with blocked mGluR-1. **D)** $1/CV^2$ analysis in LY367385. **E)** Same as A) but with GDPβS in the internal solution (pre pairing – black circles, post pairing – light green circles). **F-H)** Same as B-D) but in GDPβS. Mean ± SEM.

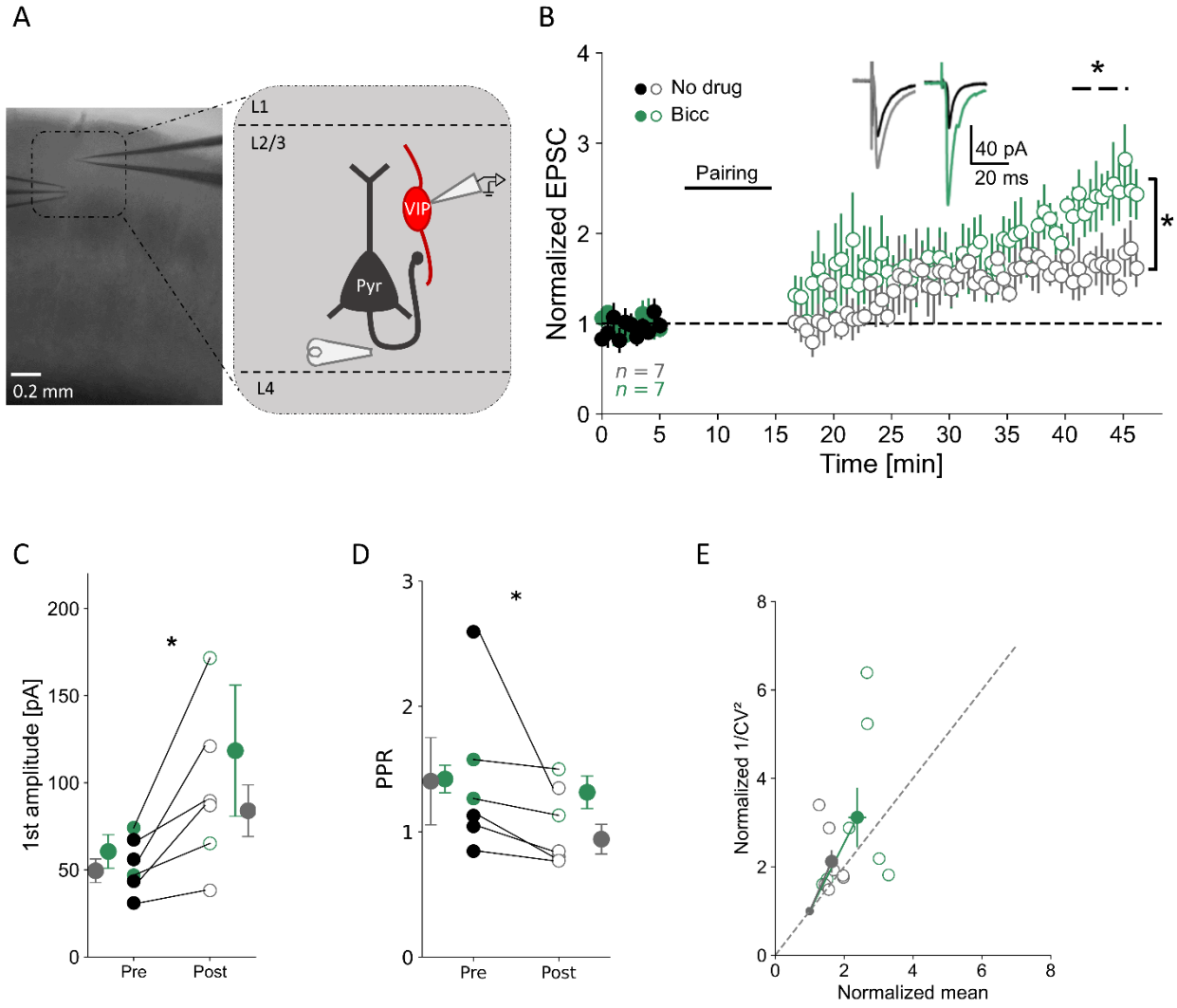
Fig. 4. Inhibition of Src-kinase abolishes LTP in VIP-INs. A) Normalized EPSC after bath application of Src-kinase inhibitor (PP2); pre pairing – black circles, post pairing – orange circles. Examples of EPSC traces for pre and post LTP induction. **B)** Averaged 1st amplitude for pre and post pairing protocol with inhibited Src-kinase. **C)** Averaged paired pulse ratio for pre and post pairing in PP2 administered condition. **D)** 1/CV^2 analysis in PP2. **E)** TRPCs are not involved in LTP as shown by time course plot of normalized EPSC before and after the pairing protocol in the presence of TRPC blocker (SKF96365, pre pairing – black circles, post pairing – purple circles). **F-H)** Same as B-D) but in SKF96365. Mean ± SEM, * $p < 0.05$.

Fig. 5. L-type VGCCs mediate synaptic plasticity at L2/3 VIP-IN excitatory inputs. A) Normalized EPSC after L-type VGCC blocker (nifedipine) application (pre pairing – black circles, post pairing – pink circles). Examples of EPSC traces for pre and post LTP induction. **B)** Averaged 1st amplitude for pre and post pairing protocol with blocked L-type VGCC activity. **C)** Averaged paired pulse ratio for pre and post pairing in the presence of nifedipine. **D)** 1/CV^2 analysis in nifedipine. Mean ± SEM.

Fig. 6. eCBs but not BDNF putatively act retrogradely on glutamatergic inputs to VIP-INs promoting presynaptic expression of LTP. A) Time course plot of normalized EPSC before and after the pairing protocol in the condition of BDNF signaling blockage with the TrkB antagonist (ANA-12, pre pairing – black circles, post pairing – brown circles). Examples of EPSC traces for pre and post LTP induction. **B)** Averaged 1st amplitude for pre and post pairing protocol in ANA-12. **C)** Averaged paired pulse ratio for pre and post pairing in ANA-12. **D)** 1/CV^2 analysis in ANA-12 conditions. **E)** Same as A) but in the presence of the CBR-1 antagonist (AM-251) inhibiting eCB retrograde signaling (pre pairing – black circles, post pairing – blue circles). **F-H)** Same as B-D) but in AM-251. Mean ± SEM, * $p < 0.05$, *** $p < 0.001$.

Fig. 7. Summary of factors implicated in LTP at VIP-IN excitatory inputs in the mouse somatosensory cortex. A) Schematic representation of a glutamatergic synapse on a VIP-IN. During LTP protocol, glutamate binding to the postsynaptic mGluR-1 presumably activates Src-kinase signaling cascade necessary for LTP induction. Additionally, membrane depolarization during LTP protocol, activates L-type VGCC leading to the postsynaptic calcium influx needed for LTP induction. eCBs act as a retrograde messenger, activating CBR-1s which in turns, induce presynaptic LTP expression. NMDAR, mGluR-5, TRPC and BDNF (TrkB) do not mediate LTP in VIP-INs. **B)** Summarized graph showing receptors and channels that are not necessary for LTP. Pharmacological interventions did not abolish LTP. **C)** Summarized plot depicting crucial factors

for synaptic plasticity at excitatory synapses on VIP-INs. Here, pharmacological agents blocked LTP.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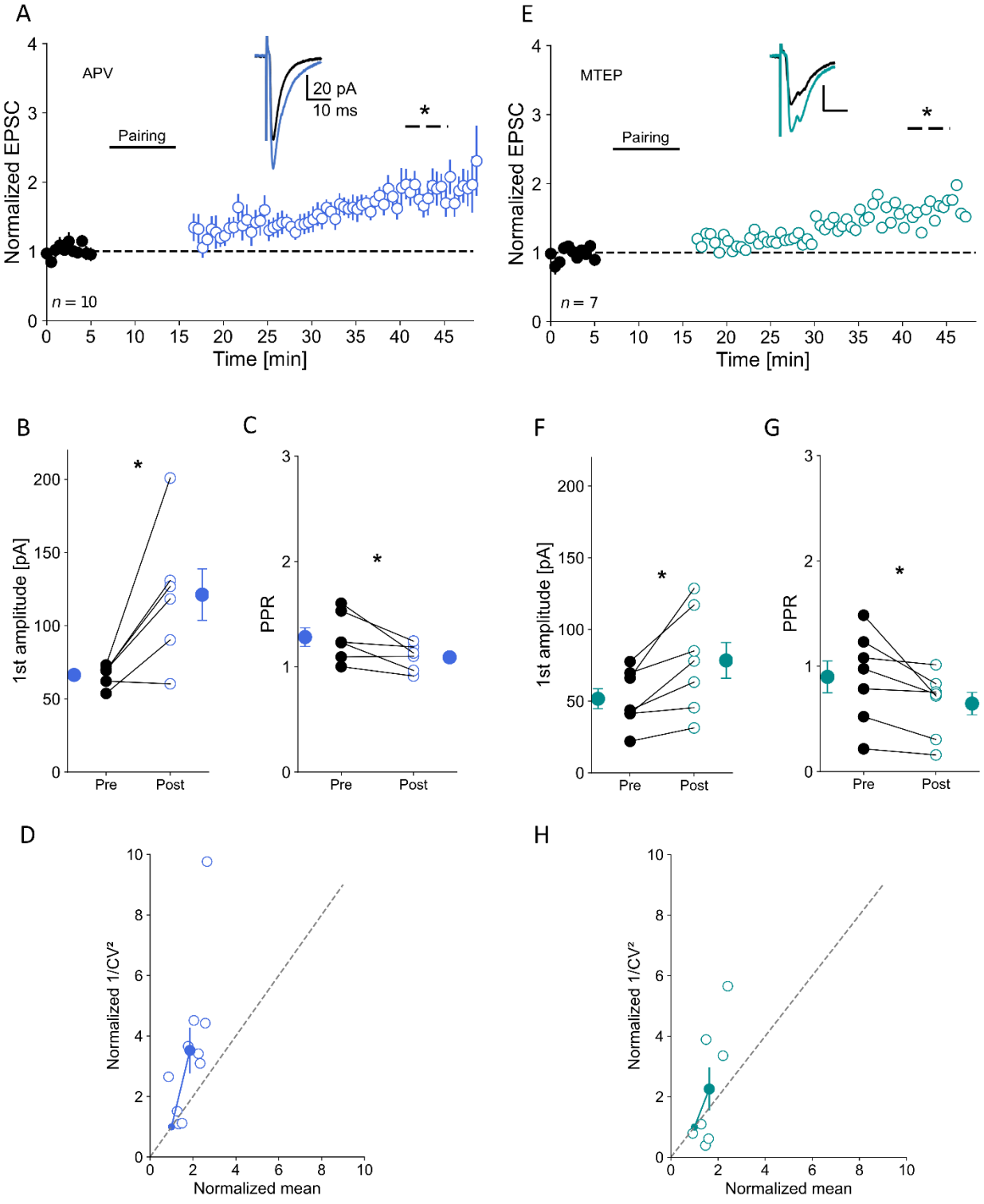
L1
L2/3
VIP
Pyr
L4
0.2 mm
B
No drug
Bicc
Pairing
40 pA
20 ms
Normalized EPSC
Time [min]
n = 7
n = 7
C
1st amplitude [pA]
Pre
Post
D
PPR
Pre
Post
E
Normalized 1/CV²
Normalized mean

Fig. 1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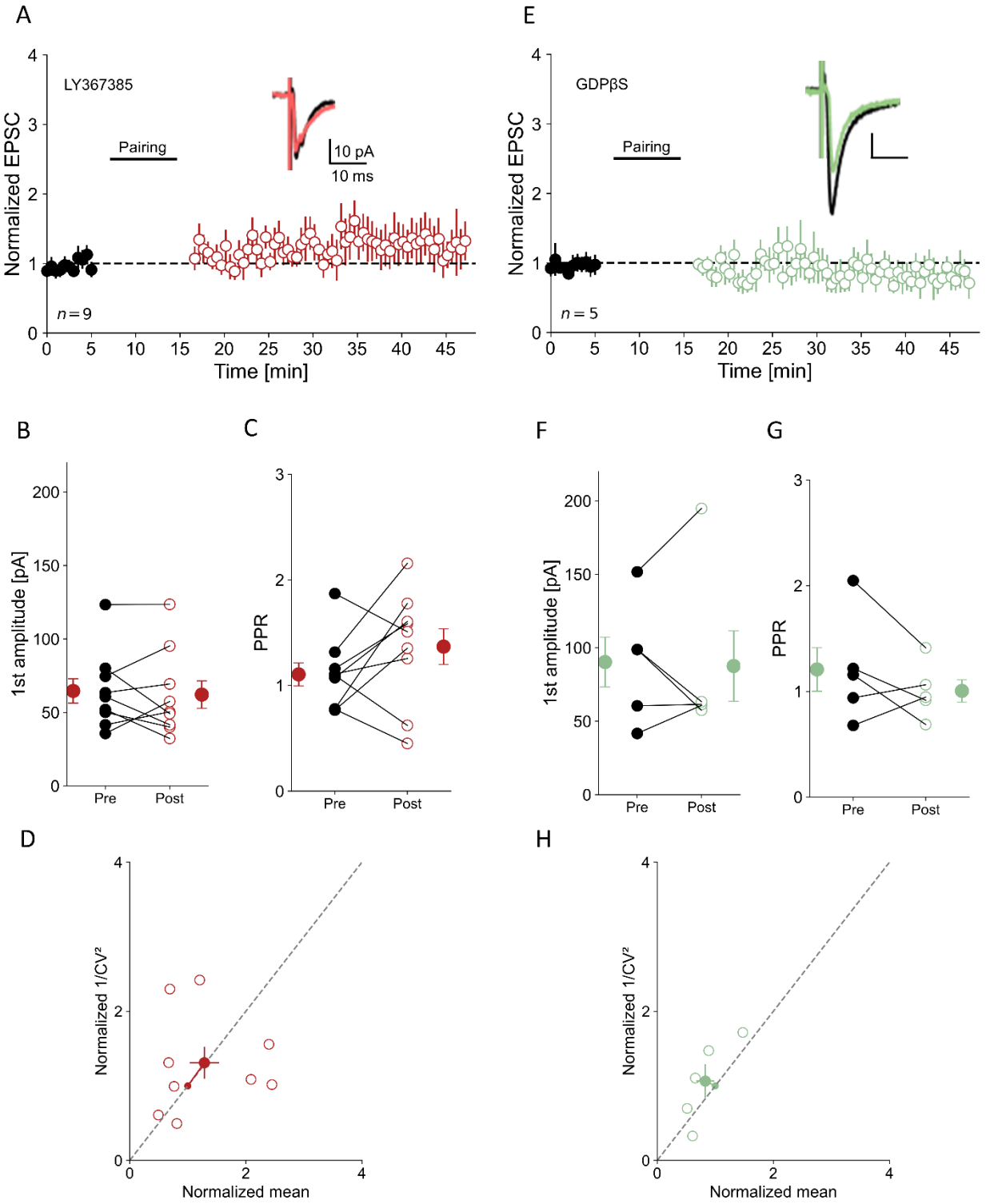
APV
Pairing
20 pA
10 ms
*
Normalized EPSC
n = 10
Time [min]
E
MTEP
Pairing
*
Normalized EPSC
n = 7
Time [min]
B
*
1st amplitude [pA]
Pre
Post
C
*
PPR
Pre
Post
F
*
1st amplitude [pA]
Pre
Post
G
*
PPR
Pre
Post
D
Normalized 1/CV²
Normalized mean
H
Normalized 1/CV²
Normalized mean

Fig. 2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LY367385
Pairing
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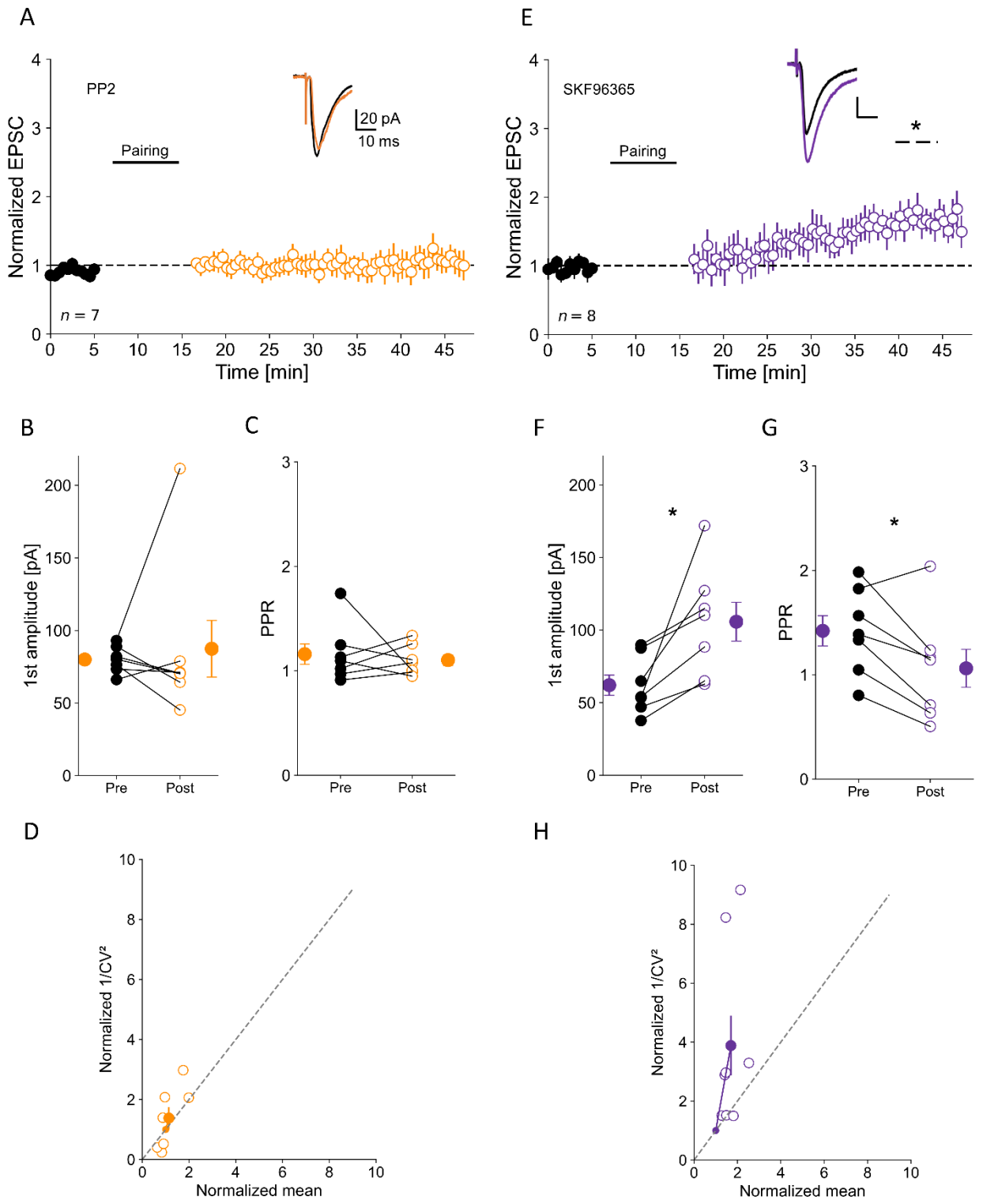
10 ms
Normalized EPSC
n = 9
Time [min]
E
GDPβS
Pairing
Normalized EPSC
n = 5
Time [min]
B
1st amplitude [pA]
Pre
Post
C
PPR
Pre
Post
F
1st amplitude [pA]
Pre
Post
G
PPR
Pre
Post
D
Normalized 1/CV²
Normalized mean
H
Normalized 1/CV²
Normalized mean

Fig. 3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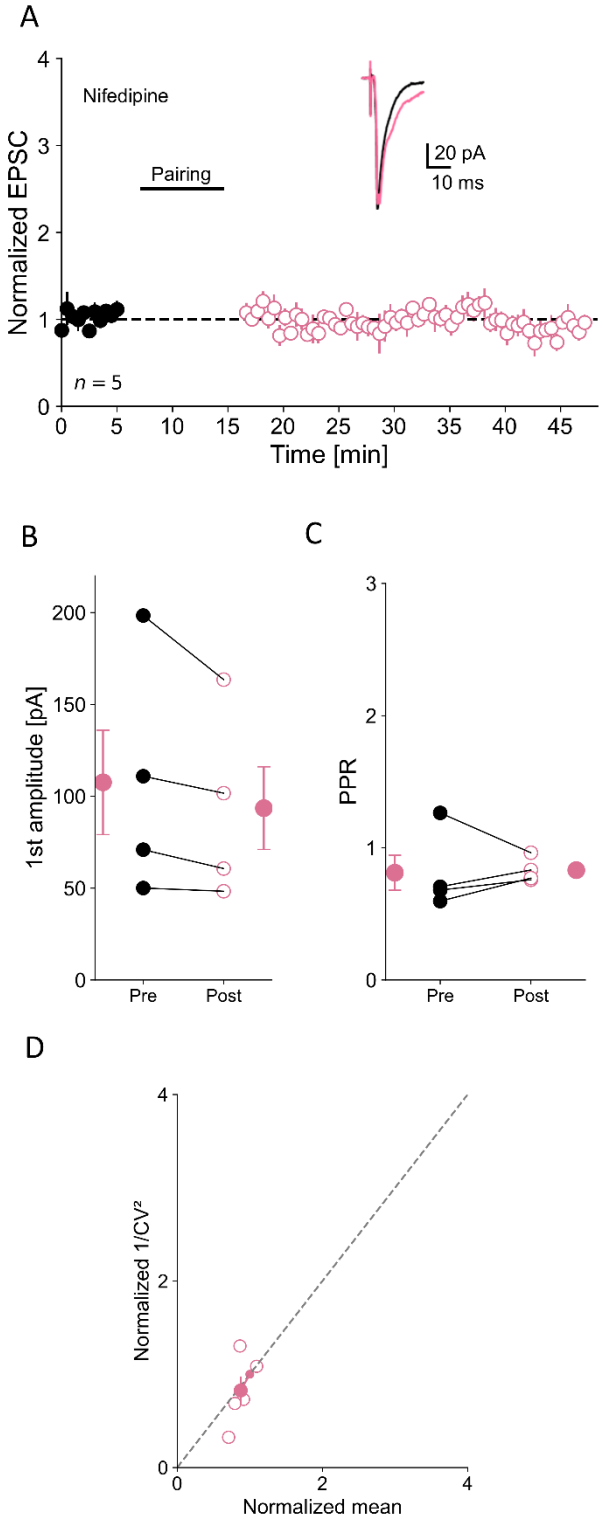
PP2
Pairing
20 pA
10 ms
Normalized EPSC
n = 7
Time [min]
E
SKF96365
Pairing
*
Normalized EPSC
n = 8
Time [min]
B
1st amplitude [pA]
Pre
Post
C
PPR
Pre
Post
F
1st amplitude [pA]
*
Pre
Post
G
PPR
*
Pre
Post
D
Normalized 1/CV²
Normalized mean
H
Normalized 1/CV²
Normalized mean

Fig. 4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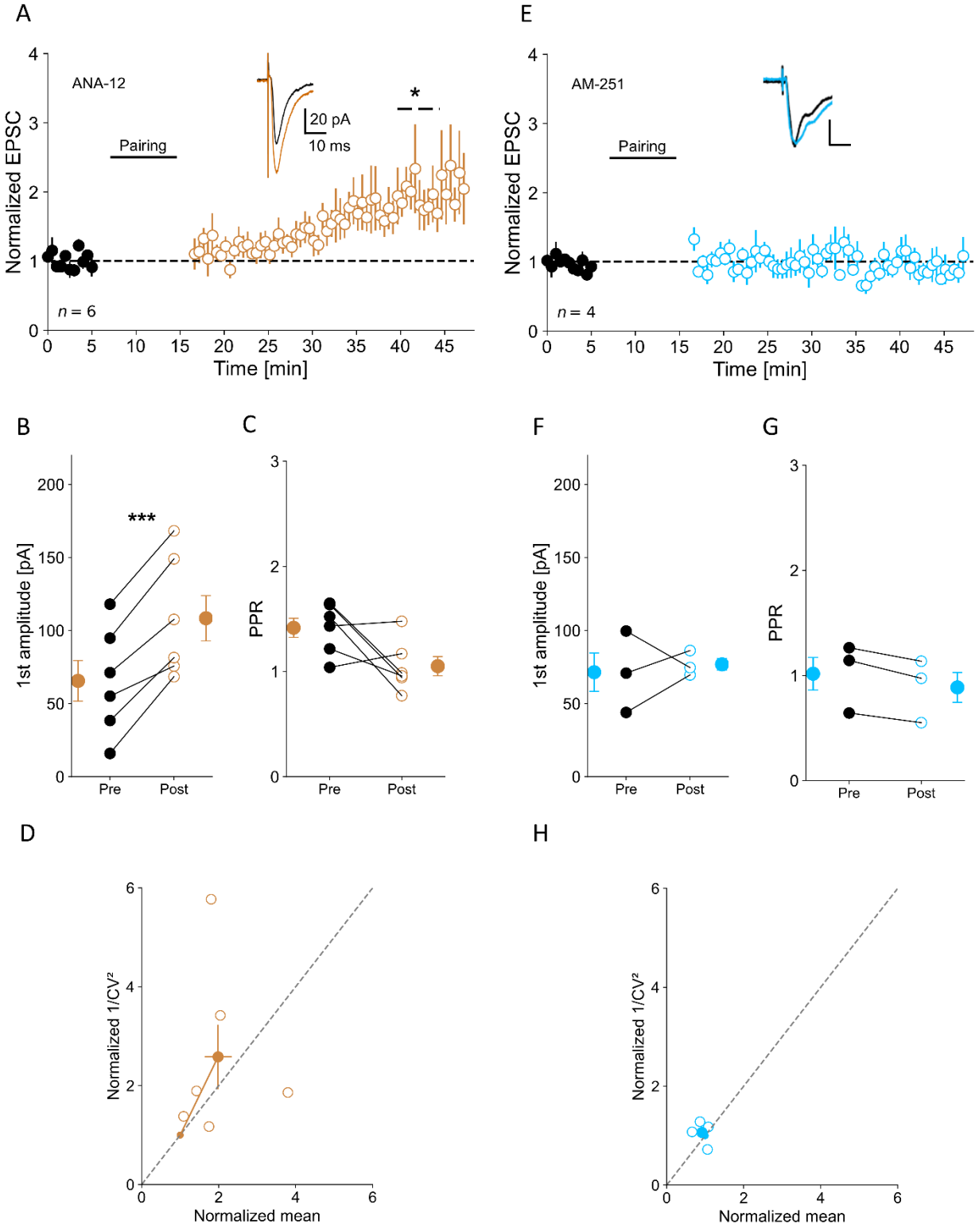
Nifedipine
Pairing
20 pA
10 ms
Normalized EPSC
n = 5
Time [min]
B
1st amplitude [pA]
Pre
Post
C
PPR
Pre
Post
D
Normalized 1/CV²
Normalized mean

Fig. 5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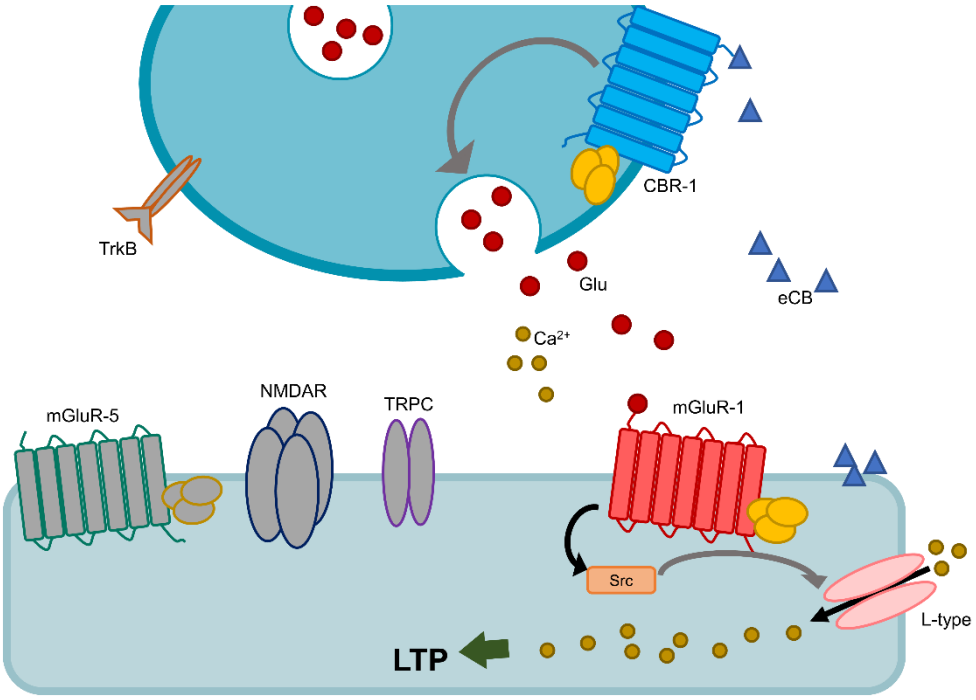
Pairing
20 pA
10 ms
*
Normalized EPSC
n = 6
Time [min]
E
AM-251
Pairing
Normalized EPSC
n = 4
Time [min]
B
***
1st amplitude [pA]
Pre
Post
C
PPR
Pre
Post
F
1st amplitude [pA]
Pre
Post
G
PPR
Pre
Post
D
Normalized 1/CV²
Normalized mean
H
Normalized 1/CV²
Normalized mean

Fig. 6

A


TrkB
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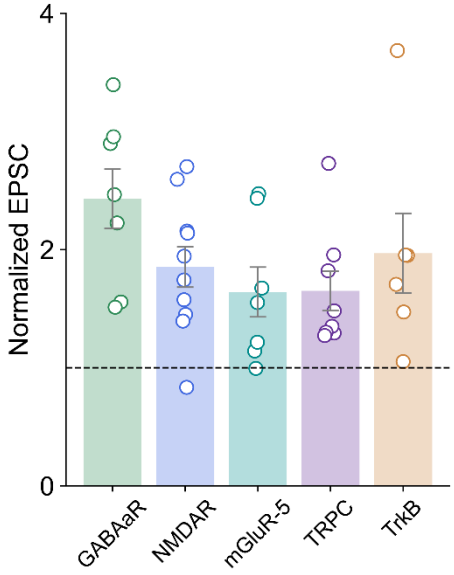
eCB
Ca2+
mGluR-5
NMDAR
TRPC
mGluR-1
Src
L-type
LTP

B


Normalized EPSC
0
2
4
GABAaR
NMDAR
mGluR-5
TRPC
TrkB

C

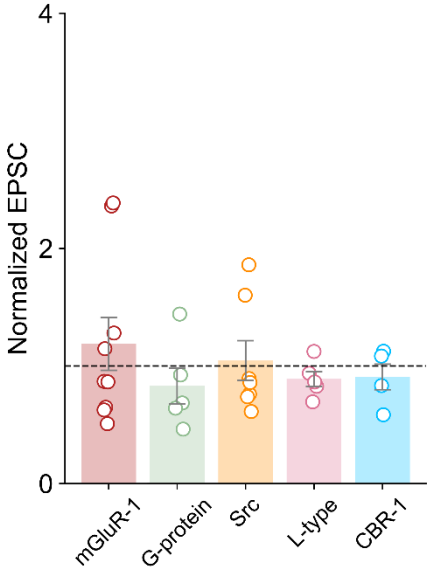

Normalized EPSC
0
2
4
mGluR-1
G-protein
Src
L-type
CBR-1

Fig. 7