Substance P induces plasticity and synaptic tagging/capture in rat hippocampal area CA2

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The hippocampal area *Cornu Ammonis* (CA) CA2 is important for social interaction and is innervated by Substance P (SP)-expressing supramammillary (SuM) nucleus neurons. SP exerts neuromodulatory effects on pain processing and central synaptic transmission. Here we provide evidence that SP can induce a slowly developing NMDA receptor- and protein synthesis-dependent potentiation of synaptic transmission that can be induced not only at entorhinal cortical (EC)-CA2 synapses but also at long-term potentiation (LTP)-resistant Schaffer collateral (SC)-CA2 synapses. In addition, SP-induced potentiation of SC-CA2 synapses transforms a short-term potentiation of EC-CA2 synaptic transmission into LTP, consistent with the synaptic tagging and capture hypothesis. Interestingly, this SP-induced potentiation and associative interaction between the EC and SC inputs of CA2 neurons is independent of the GABAergic system. In addition, CaMKIV and PKMζ play a critical role in the SP-induced effects on SC-CA2 and EC-CA2 synapses. Thus, afferents from SuM neurons are ideally situated to prime CA2 synapses for the formation of long-lasting plasticity and associativity.

long-term potentiation | CA2 region | Substance P | synaptic tagging | social memory

The hippocampus is a temporal lobe structure important for the formation of spatial and episodic memories. The hippocampus consists of *Cornu Ammonis* (CA) areas CA1, CA2, and CA3, containing pyramidal neurons and the dentate gyrus, containing granule cells. The CA2 area is a small region interposed between CA1 and CA3. Although its function remained unknown for many years, CA2 has been recently shown to play a critical role in social memory (1, 2) and aggressive behavior (3). Morphologically distinguishable from CA1 neurons based on their larger cell bodies, CA2 pyramidal neurons receive direct inputs from entorhinal cortical (EC) layer II (LII) and CA3 neurons. It is notable that the Schaffer collaterals (SCs) from CA3 neurons form synapses with CA2 neurons that do not express typical activity-dependent long-term potentiation (LTP) (4, 5), a property that is very different than EC-CA2 synapses. This difference is likely a result of the local existence of specific calcium-binding proteins (6), regulator of G protein signaling RGS14 (7), and the complex inhibitory circuits in CA2 compared with the neighboring CA1 and CA3 areas (8, 9).

The CA2 area also receives a number of projections from the hypothalamic supramammillary nucleus (SuM), which express various neuroactive peptides, such as cholecystokinin, Substance P (SP), and vasoactive intestinal polypeptide (8, 9). The SP-expressing SuM fibers terminate specifically at the area CA2 (10). SP is an 11-aa neurotransmitter (11) that has neuromodulatory properties and has been implicated in physiology, disease, and pain (12). It has been reported earlier that bath application of SP modulates synaptic transmission in mouse hippocampal SC-CA1 synapses (13) and enhances the local inhibitory responses in the hippocampus by influencing release of GABA by acting on neurokinin-1 (NK1) receptors (14). SuM activity has been demonstrated with stress (15, 16), but it is unclear whether SP is released from SuM terminals to CA2. In general, the role of SP in regulating plasticity in CA2 pyramidal neurons has not yet been studied.

Late associativity is a unique feature of synaptic networks that leads to strengthening of synaptic inputs that are originally not sufficiently activated to form LTP (17, 18). This process can be induced by learning events that are strong enough to form LTP of synaptic transmission, and the phenomenon is known as synaptic tagging and capture (STC) (17, 19). In the present study, we investigated whether SP can initiate plasticity in SC and EC synapses of rat hippocampal area CA2. We observed that bath application of SP (5 μM) induced a long-lasting slow-onset potentiation of the two different afferent inputs of CA2 pyramidal neurons. Interestingly, the SP-induced plasticity-related proteins (PRPs) from SC-CA2 synapses were able to prime the EC-CA2 synaptic inputs in an STC-dependent manner. In addition, we showed that CaMKIV and PKMζ play critical roles in the SP-induced synaptic plasticity and associativity within the area CA2.

Statistics

The average values of the slope function of field excitatory postsynaptic potentials (fEPSPs) and excitatory postsynaptic currents (EPSCs) per time point (20, 21) were analyzed by Wilcoxon signed-rank test (henceforth "Wilcoxon test") when compared within the same group (before and after induction of synaptic plasticity). The Mann–Whitney *U* test was applied when compared between groups. A Student's *t* test at the $P < 0.05$ significance level was used for the analysis of RT-PCR (22) and Western blot results (23, 24). Detailed descriptions of statistical analysis of each experiment are provided in *SI Methods* and Dataset S1.

Significance

The hippocampal area *Cornu Ammonis* (CA) CA2 is a small region interposed between CA1 and CA3. For a long time, there has been a lack of information on the CA2 area's role in memory formation. This area is innervated by supramammillary axonal fibers that are rich with Substance P (SP), which acts as a neurotransmitter and neuromodulator. We show that SP induces an NMDA receptor- and protein synthesis-dependent potentiation of CA2 synapses that requires kinases such as CaMKIV and PKMζ. The SP-induced effects on Schaffer collateral-CA2 synapses transform entorhinal cortical-CA2 short-term potentiation into long-term potentiation, thereby expressing synaptic tagging and capture, an associative property of neuronal populations that engage in consolidation.

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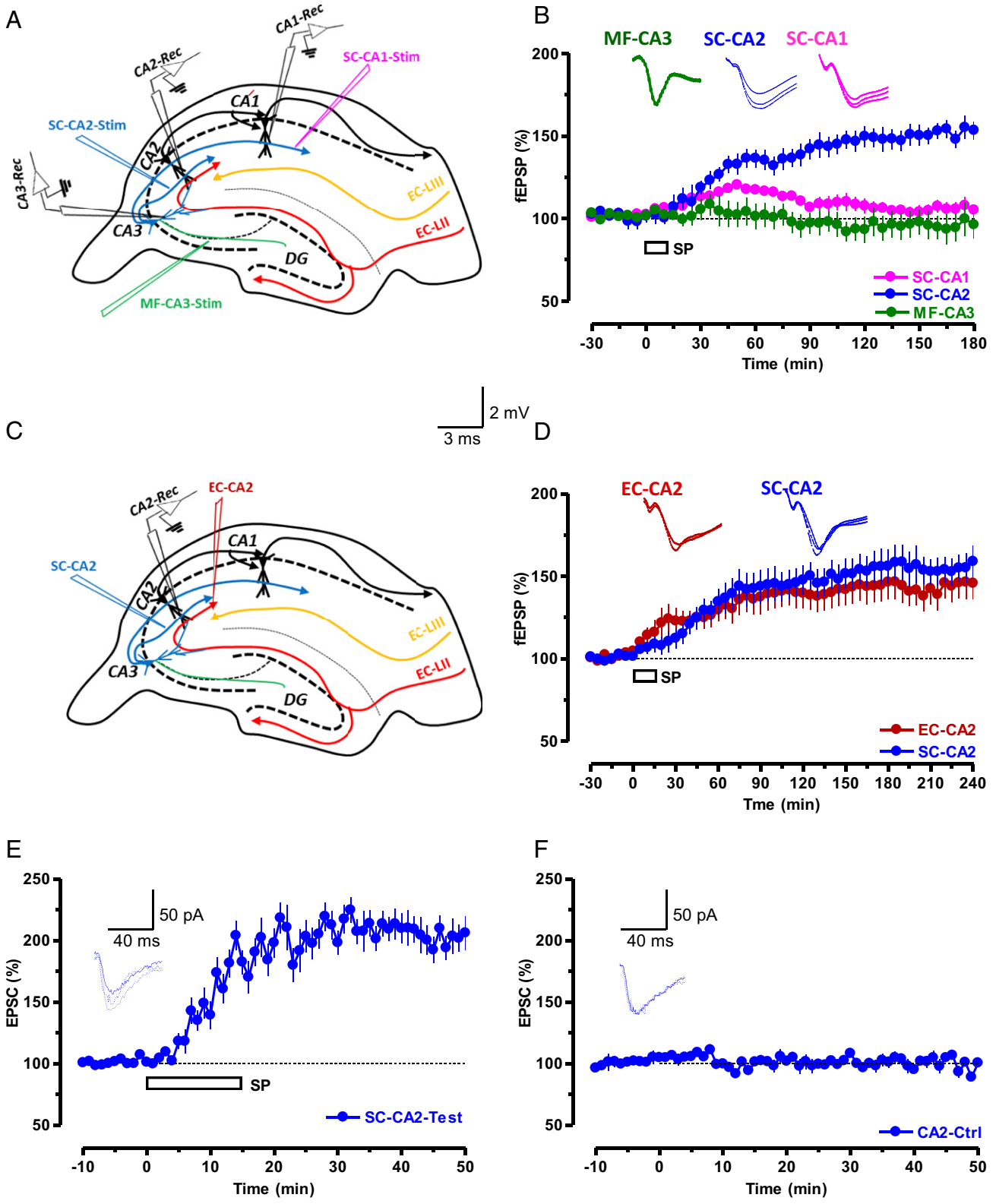

Fig. 1. SP induces long-lasting potentiation of synaptic transmission in CA2 neurons. (*A*) The schema shows the location of electrodes for stimulation of synaptic inputs MF-CA3 (green), SC-CA2 (blue), SC-CA1 (pink), and the recording sites of fEPSPs within the hippocampal CA3, CA2, and CA1 areas. (*B*) Bath application of 5 μM SP induced a slow-onset, long-lasting potentiation in SC-CA2, but not in MF-CA3 and SC-CA1 synaptic inputs ($n = 5$). (*C*) The schema shows the location of electrodes for the stimulation of SC-CA2 (blue) and EC-CA2 (red) synaptic inputs and the recording sites of fEPSPs within the hippocampal CA2 area. (*D*) Bath application of SP for 15 min after a stable baseline of 30 min induced a synaptic potentiation in SC-CA2 and EC-CA2 synaptic inputs ($n = 10$). (*Insets*) Representative fEPSPs 15 min before (closed line), 60 min after (dotted line), and 180 min after (hatched line) SP application. (*E*) Whole-cell voltage-clamp recording of EPSCs with the application of SP (5 μM) induced a slow-onset potentiation in SC-CA2 ($n = 10$). (*F*) Control experiments indicated the stability of the recordings ($n = 6$). (*Insets*) Representative EPSC 5 min before (closed line), 30 min after (dotted line), and 50 min after (hatched line) SP application. Calibration bars are 2 mV/3 ms for fEPSP and 50 pA/40 ms for EPSC traces.

Results

SP Induces NMDA Receptor- and Protein Synthesis-Dependent Long-Lasting Potentiation in the Hippocampal Area CA2. First, we evaluated the distribution of SP receptors (i.e., NK1) in the CA1, CA2, and CA3 regions and observed a higher level of NK1 transcripts in CA2 and CA3 compared with CA1 (Fig. S1). We then investigated if direct application of SP can induce plasticity in areas CA1, CA2, and CA3. By using fEPSP recordings (Fig. 1*A*) from the CA1, CA2, and CA3 regions at the same time, we observed that bath application of SP (5 μM) for 15 min resulted in a slowly developing long-lasting potentiation of synaptic transmission only in area CA2 (Fig. 1*B*, blue circles). Statistically significant potentiation was observed in SC-CA2 synapses starting from the 30th minute (Wilcoxon test, $P = 0.04$) and lasting as long as 3 h (180 min). Neither SC-CA1 (Fig. 1*B*, pink circles) nor mossy fiber (MF)-CA3 (Fig. 1*B*, green circles) synapses showed statistically significant potentiation at any recorded time points. An earlier study showed that application of an antagonist of adenosine A_1 receptor, 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX), induced plasticity only in area CA2 (25). We confirmed these findings by using a similar simultaneous stimulation and recording of CA2 and CA1 regions of hippocampal slices (Fig. S2*A*). Indeed, bath application of DPCPX (10 nM) for 15 min induced a slowly developing potentiation in SC-CA2 (Fig. S2*C*, red circles) and EC-CA2 (Fig. S2*C*, blue circles) synapses, but not in the CA1 area (pink circles; Fig. S2*C*). Baseline responses recorded without DPCPX remained stable in all synaptic inputs (Fig. S2*B*). In addition, electrophysiological investigation by using paired-pulse stimulation between the two inputs (50-ms interstimulus interval; Fig. S2*D*) confirmed that we recorded from independent SC and EC synaptic inputs in CA2.

As the focus of the study was on SC-CA2 and EC-CA2, we restricted future recordings to these synapses by using two-pathway experiments (Fig. 1*C*). It has been noted previously that the SC synapses in area CA2 are resistant to the induction of activity-dependent plasticity such as LTP, whereas EC synapses are not (4, 5). The results presented in Fig. S3 are consistent with earlier findings (4, 5) and provide additional evidence that we were studying the exact CA2 synapses. We further explored the effects of SP on EC-CA2 and SC-CA2 synaptic inputs (Fig. 1*D*) and noticed that SP could induce a slowly developing potentiation in SC (from the 40th minute; Wilcoxon test, $P = 0.008$) and EC synapses (from the 30th minute; Wilcoxon test, $P = 0.007$) that lasted for 4 h (Fig. 1*D*, blue and red circles). The observed potentiation was found to be NMDA receptor- and protein synthesis-dependent (Fig. S4 *A*–*C*), and the potentiation was completely abolished in the presence of SP receptor (i.e., NK1) antagonist L-733060 (5 μM; Fig. S4*D*). Nonetheless, there is a reasonable possibility that extracellular recording from area CA2 may not be appropriate to monitor fEPSPs from SC and EC that are purely from CA2 because distal dendrites of CA3 pyramidal neurons are very likely to be stimulated with the stimulating electrodes in area CA2. To check this possibility, we conducted whole-cell patch-clamp recordings from CA2 pyramidal neurons (Fig. 1 *E* and *F*). Baseline stimulation in SC-CA2 for 10 min followed by bath application of SP for 15 min resulted in a statistically significant potentiation from the seventh minute that lasted as long as 60 min (Wilcoxon test, $P = 0.005$; Fig. 1*E*, blue circles), whereas control stimulation without SP resulted in relatively stable potentials throughout the experimental period (Fig. 1*F*, blue circles).

Test Stimulation Is Required for the Expression of SP Potentiation. We have reported earlier that test stimulation is critical for expressing dopamine or D1/D5 receptor agonist-induced potentiation (26–28). Suspending test stimulation during dopamine or D1/D5 agonist application prevented the expression of plasticity in those inputs, and we had used those silenced inputs to study the STC interactions. Thus, we tested whether test stimulation during SP application was necessary to express SP-mediated plasticity. A series of experiments was conducted to test the requirements of test stimulations in SP-induced plasticity in SC-CA2 and EC-CA2 synaptic inputs (Fig. 2). In Fig. 2 *A* and *B*, SP was applied to the bath medium, but test pulses were suspended in EC-CA2 (Fig. 2*A*, red circles) or in SC-CA2 (Fig. 2*B*, blue circles) for the subsequent hour (total of 60 min including the SP application period of 15 min). SP-induced potentiation was not observed in the synaptic inputs that did not receive test stimulation during SP application. The slow onset potentiation observed in SC-CA2 (Fig. 2*A*, blue circles) and EC-CA2 (Fig. 2*B*, red circles) was significantly different from the 30th and 20th minutes onward compared with their own baselines (Wilcoxon test, $P = 0.017$ and $P = 0.017$). In Fig. 2*C*, SP was applied to the bath medium, but, in both EC and SC synaptic inputs, the baseline recordings were suspended at the time of SP application and for the subsequent 1 h. In both synaptic inputs, the potentials remained stable at baseline levels, and there was no significant potentiation compared with the respective baseline values before drug application (Wilcoxon test, $P > 0.05$). The control experiments displayed in Fig. 2*D* used the same experimental design as in Fig. 2*C*, but the baseline recordings were suspended for 1 h without SP application. The baseline values in EC-CA2 and SC-CA2 inputs showed stable recordings before and after suspending the test stimulation (Fig. 2*D*, red and blue circles). In short, test stimulation during SP application is critical for the expression of long-lasting plasticity in SC and EC synaptic inputs.

SP-Induced Plasticity Expresses STC. According to the STC paradigm, application of a weak stimulation such as a single tetanization [weak tetanization (WTET); *Methods*] or repeated tetanization [strong tetanization (STET); *Methods*] in the presence of protein synthesis inhibitors results in only a transient form of plasticity, early LTP. However, this process is able to "mark" the synapses to set "synaptic tags" (17, 18). The synaptic tags can then presumably capture plasticity factors from nearby "strong inputs" [inputs that express protein synthesis-dependent late LTP (L-LTP)], eventually expressing long-lasting plasticity (17, 18). We conducted a series of experiments within the STC framework to determine whether SP could cause the expression of PRPs that contribute to the potentiation of "tagged synapses." Initially, STC was studied by using a strong-before-weak paradigm (SBW). Here, SP-induced potentiation was considered strong because of its protein synthesis dependency and its long-lasting nature. To study STC in the framework of SBW, a 30-min stable baseline was recorded from SC-CA2 and EC-CA2 inputs (Fig. 3*B*, blue and red circles) before the bath application of SP for 15 min. The test stimulation in EC-CA2 (Fig. 3*B*, red circles) was suspended during SP application period and for the subsequent 1 h, whereas synaptic responses in SC-CA2 (Fig. 3*B*, blue circles) was recorded continuously. Consistent with the findings depicted in Fig. 2*A*, SC-CA2 expressed statistically significant potentiation starting from the 40th minute (Wilcoxon test, $P = 0.013$) whereas no potentiation was observed in EC-CA2. An early-LTP protocol (i.e., WTET) was delivered to EC-CA2 (Fig. 3*B*) 30 min after resuming the baseline stimulation in this input. Interestingly, we observed an expression of L-LTP at weakly stimulated EC-CA2 synaptic input (Fig. 3*B*, red circles) that, without SP, would have decayed back to the baseline level (Fig. 3*A*). The next step was to test whether continuous stimulation of SC was required during SP application to prime EC-CA2 synapses to express late plasticity or if SP application during the suspension of stimulation was sufficient. As shown in Fig. S5*A*, stopping the test stimulation entirely in SC-CA2 (Fig. S5*A*, blue circles) 60 min after SP application still primed the EC-CA2 synapses to express L-LTP (Fig. S5*A*, red circles). In contrast, suspending test stimulation in SC-CA2 during SP application and then for as long as 60 min did not prime the EC-CA2 to express L-LTP (Fig. S5*B*, red circles). Similarly, sus-

pending test stimulation in SC-CA2 during SP application and then as long as 60 min, followed by the application of STET 30 min after resuming the test stimulation, also failed to induce plasticity in this input (Fig. S5*C*, blue circles). In this experiment, the EC-CA2 input received test stimulation throughout the recording period of 240 min and displayed statistically significant potentiation starting from the 40th minute throughout the recording period (Fig. S5*C*, red circles; Wilcoxon test, $P = 0.027$).

Next, we confirmed the STC experiments by using a strong-before-strong (SBS) paradigm (29) using the same experimental design as in Fig. 3*B*. However, instead of early LTP, L-LTP was induced in EC-CA2 in the presence of a protein synthesis inhibitor, anisomycin (ANI; 25 μM) or emetine (EME; 20 μM), that resulted in an L-LTP in the EC-CA2 input (Fig. 3 *D* and *F*, red circles). SC-CA2 showed statistically significant potentiation from the 30th minute to the end of the recording (Fig. 3 *D* and *F*, blue circles; Wilcoxon test, $P = 0.017$ and $P = 0.016$, respectively), and EC-CA2 showed statistically significant potentiation immediately after STET (Fig. 3 *D* and *F*, red circles; Wilcoxon test, $P = 0.012$ and $P = 0.003$, respectively). In both cases, the drug was applied 20 min before and continued for 40 min after the first tetanization of STET. The control experiments depicted in Fig. 3 *C* and *E* used the same experimental design as of Fig. 3 *D* and *F* except that SC-CA2 was not recorded. Induction of L-LTP in the presence of ANI (Fig. 3*C*) or EME (Fig. 3*E*) in these experimental conditions resulted in only early LTP. Statistically significant potentiation was present only up to the 205th minute in Fig. 3*C* (Wilcoxon test, $P = 0.04$) and up to the 215th minute in Fig. 3*E* (Wilcoxon test, $P = 0.018$).

In short, SP-induced long-lasting plasticity participates in late associative processes reminiscent of STC.

CaMKIV and PKMζ Are Required for SP-Induced Plasticity and Associativity in Area CA2. It has been reported that CaMKIV and PKMζ can play important roles in maintaining long-term plasticity and STC in hippocampal area CA1 (30, 31). We investigated whether these molecules may play a role in SP-induced plasticity and associativity in area CA2. It has also been reported that many genes are robustly activated or down-regulated at the mRNA level upon cutting slices, and that these changes can persist for 6 h (32). To exclude this possibility, we have first determined the mRNA level in area CA2 from naïve rat tissue for reference. Expression of mRNA in naïve rat hippocampus and control hippocampal slices did not show any significant difference (Fig. S6; more details provided in *Methods*).

Next, we determined the expression levels of CaMKIV and PKMζ mRNAs in area CA2 before and after the establishment of SP-induced potentiation (Fig. S6). We noticed significantly higher expression of CaMKIV and PKMζ mRNA after SP application ($P < 0.05$; Fig. S6) compared with control (unstimulated) slices and naïve (i.e., not sliced) CA2 tissues. These findings motivated us to test whether pharmacological inhibition of CaMKIV or PKMζ abolishes SP-induced long-lasting plasticity. We tested this notion by inhibiting CaMKIV with KN-93 (10 μM) and inhibiting PKMζ with antisense oligodeoxynucleotides (20 μM). Coapplication of KN-93 along with SP completely abolished SP-induced potentiation in SC-CA2 and EC-CA2 inputs (Fig. 4*A*, red and blue circles), whereas a nonactive version of the drug KN-92 (10 μM) did not prevent SP-induced potentiation (Fig. 4*B*, red and blue circles). Statistically significant potentiation was observed in SC and EC-CA2 inputs from the 10th and 35th minutes (Wilcoxon test, $P = 0.046$ and $P = 0.03$, respectively), which lasted for 240 min. Similarly, continuous PKMζ inhibition by antisense oligodeoxynucleotides (20 μM) during the incubation and the entire recording period prevented SP-induced potentiation (Fig. 4*D*, red and blue circles), whereas a control scrambled version left SP-induced potentiation intact (Fig. 4*E*, red and blue circles). The potentiation observed in SC- and EC-CA2 inputs showed statistical significance starting from the 70th and 50th minutes onward (Wilcoxon test, $P = 0.018$ and $P = 0.03$, respectively). The knockdown experiments of PKMζ by antisense and scrambled oligodeoxynucleotides were validated by Western blot analysis (Fig. S7). We further quantified the CaMKIV and PKMζ protein levels by Western blot analysis and Fig. 4 *C*, *a* and *b*, and *F*, *a* and *b*, show compelling evidence that phosphorylated CaMKIV and total PKMζ are significantly increased in area CA2 after the application of SP.

Previous studies have shown that CaMKIV is activated by phosphorylation in the CA1 area of hippocampus after LTP induction (33). We showed a change in the phosphorylation activity of CaMKIV in the area CA2, rather than the total amount of proteins after SP-induced potentiation. The Western blot analysis showed that total CaMKIV level did not change after SP treatment, but phosphorylated CaMKIV (p-CaMKIV) showed a significant increase after SP treatment in area CA2. We normalized p-CaMKIV protein expression with the respective total CaMKIV protein expression to measure the change in the fraction of total proteins that have been phosphorylated and thereby activated. SP-induced CaMKIV activity by phosphorylation may increase the expression of CaMKK enzymes, which are known to phosphorylate the CaM kinase cascade (34).

On the contrary, new PKMζ proteins are synthesized during LTP, as an elevated level of Ca^{2+} can activate different types of kinases (such as CaMKII and PKA) that can remove the translational block of PKMζ mRNA and subsequently synthesize new proteins (35). Thus, for PKMζ protein expression, we preferred to show the total amount of proteins that were synthesized during SP-induced potentiation.

SP-Induced Plasticity and Associativity in Area CA2 Is Independent of the GABAergic Transmission. Many different classes of interneurons strongly express SP in all fields of the hippocampus (10), and the NK1 receptor is expressed by several different interneuron populations in the hippocampus (36). In this context, we cannot rule out the possibility of the release of SP by interneurons in the hippocampal slices by electrical stimulation in SC-CA2 and EC-CA2 synaptic inputs. Nevertheless, we have repeated some of the critical experiments in the presence of $GABA_A$ or $GABA_B$ receptor antagonists to determine whether GABAergic transmission is required for SP-induced potentiation. The first of this series of experiments displayed in Fig. 1*D* was repeated in the presence of an inhibitor of $GABA_A$ receptors, picrotoxin (PTX; 100 μM), and $GABA_B$ receptors CGP55845 (2 μM), applied together during the entire incubation and recording period. SP-induced potentiation was intact in SC-CA2 and EC-CA2 synaptic inputs (Fig. 5*A*, blue and red circles) irrespective of the complete blockade of GABAergic transmission. Both synaptic inputs showed statistically significant potentiation from the 20th and 30th minutes (Wilcoxon test, $P = 0.027$ and $P = 0.046$) onward, and lasted to the end of the experiment. Second, we repeated the STC experiments displayed in Fig. 3 *B* and *F* in the presence of inhibitors of $GABA_A$ and $GABA_B$ receptors. Interestingly, even during the continuous inhibition of GABAergic transmission, STC initiated by SP was successfully established within the framework of tetanus-induced potentiation in SBW (Fig. 5*B*) or SBS (Fig. 5*C*) configurations. SP-induced potentiation in SC-CA2 showed statistically significant potentiation from the 25th and 15th minutes in Fig. 5 *B* and *C* (blue circles; $P = 0.018$ and $P = 0.046$, respectively) and remained stable from the 120th minute to the end of the recording. Statistically significant LTP was expressed in EC-CA2 immediately after the application of WTET ($P = 0.018$; Fig. 5*B*) or STET ($P = 0.03$; Fig. 5*C*).

In short, SP-induced plasticity and associativity in area CA2 is independent of GABAergic transmission.

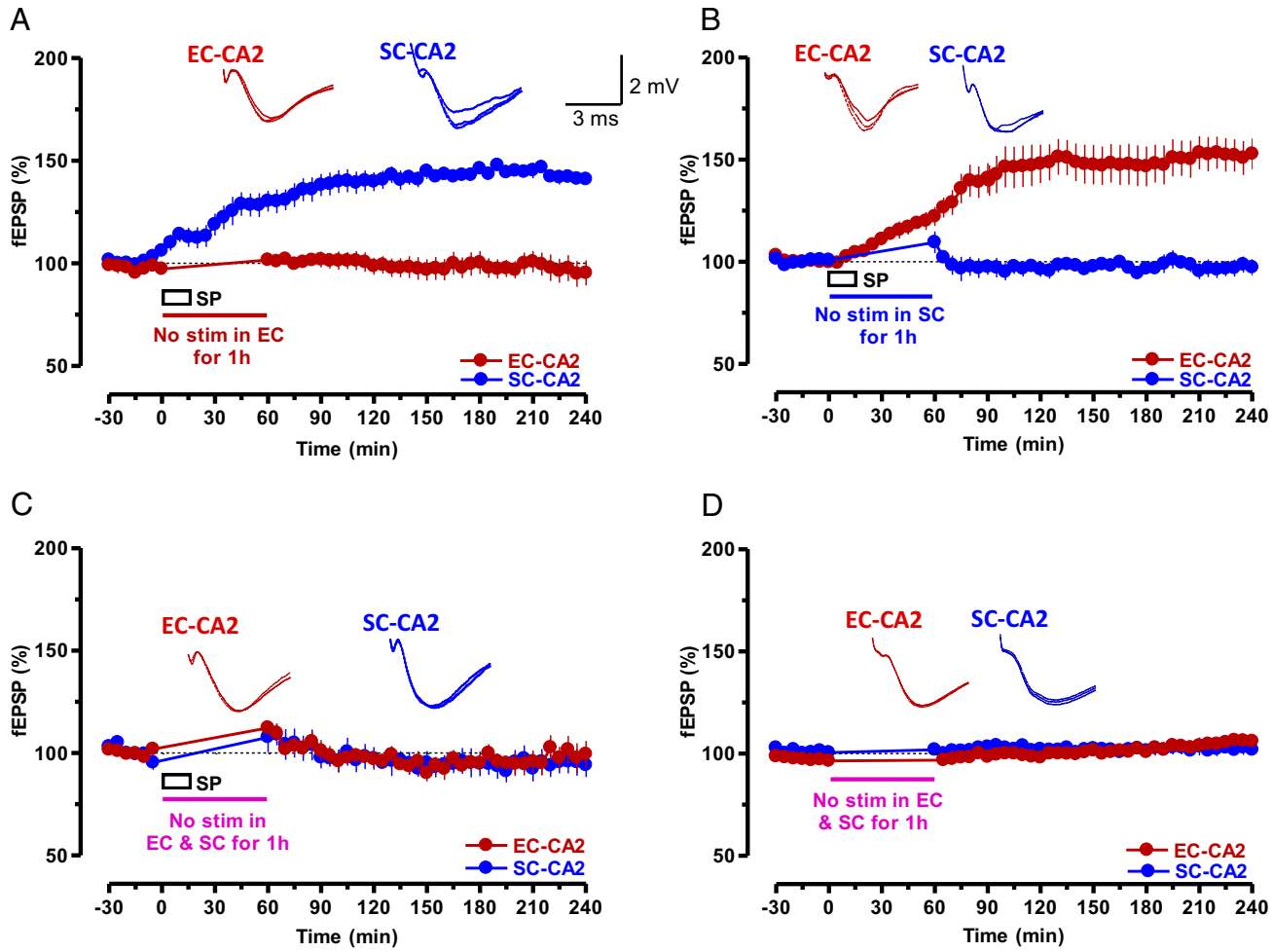

Fig. 2. Expression of SP-mediated potentiation requires test stimulation. (*A*) The potentiation by SP requires test stimulations and is input-specific. Only SC-CA2 synapses, but not the EC-CA2 synapses, showed potentiation when the EC-CA2 test stimulation was suspended at the time of SP application and for a subsequent 1 h ($n = 8$). (*B*) In a reverse scenario, i.e., suspension of SC-CA2 test stimulation during and 45 min after drug application, no potentiation was observed in SC-CA2, unlike the case for EC-CA2 synapses, in which potentiation was expressed ($n = 7$). (*C*) Suspended baseline stimulation of SC-CA2 and EC-CA2 synaptic inputs at the time of SP application and for a subsequent 1 h prevented potentiation in either synaptic input ($n = 8$). (*D*) A control experiment showing no potentiation in SC-CA2 and EC-CA2 synaptic inputs in response to the suspension of baseline stimulation and in the absence of drug application ($n = 6$). Representative fEPSP traces 15 min before (closed line), 95 min after (dotted line), and 180 min after (hatched line) SP application are depicted. Calibration bars for fEPSP traces in all panels are 2 mV/3 ms.

Discussion

The present study provides compelling evidence that activation of NK1 receptors enables SC-CA2 synapses to respond to synaptic activation by expressing synaptic potentiation. SP in conjunction with NMDA-receptor activation induces gene expression that facilitates synaptic changes that eventually lead to long-lasting potentiation. SP shows preferential binding to the ligand-binding site of NK1 receptors (37). Binding of SP to NK1 receptors leads to activation of adenylyl cyclase, hydrolysis of phosphoinositides, mobilization of intracellular calcium ions, and activation of downstream effector molecules such as PKA, PKC, and MAPK (38), all of which have been associated with the expression of LTP (39).

Kinases such as CaMKIV and PKMζ have been reported to play important roles in maintaining STC in hippocampal area CA1 (30, 31). It has been proposed earlier that CaMKII can mediate the setting of synaptic tags while CaMKIV acts as a PRP (30, 40). Similarly, tagging of PKMζ from a strongly tetanized input to a weakly tetanized input is critical for the expression of STC in CA1 pyramidal neurons (31). In addition, we have recently reported that metaplastic activation of PKMζ can rescue the plasticity and associativity that is degraded under neurodegenerative conditions (41).

We found that the levels of CaMKIV and PKMζ (*PRKCZ*) transcripts were significantly increased within the area CA2 after SP application, indicating that area CA2 also shares similar molecular pathways as CA1 during the establishment of SP-induced potentiation and its associativity. It should be noted that the CaM kinase inhibitor used in this study, KN-93, has a broad spectrum of specificity for inhibiting CaM kinases (30). A low concentration of KN-93 (1 μM) is enough to impair synaptic plasticity in brain slices (42). CaMKII (with a K_i of 370 nm) can be effectively blocked by a low concentration of KN-93 with much less inhibition on CaMKIV (43, 44). The study by Redondo et al. (30) reported that a general CaMK inhibitor such as KN-93 at a low dose inhibits CaMKII, thus specifically impairing the synaptic tag setting process, whereas a higher concentration (10 μM) of KN-93 blocks tag setting, synthesis, and availability of PRPs. Thus, the high concentration of KN-93 used in the present study to prevent SP-induced potentiation in area CA2 must have interfered with tag setting and PRPs (here CaMKIV) synthesis.

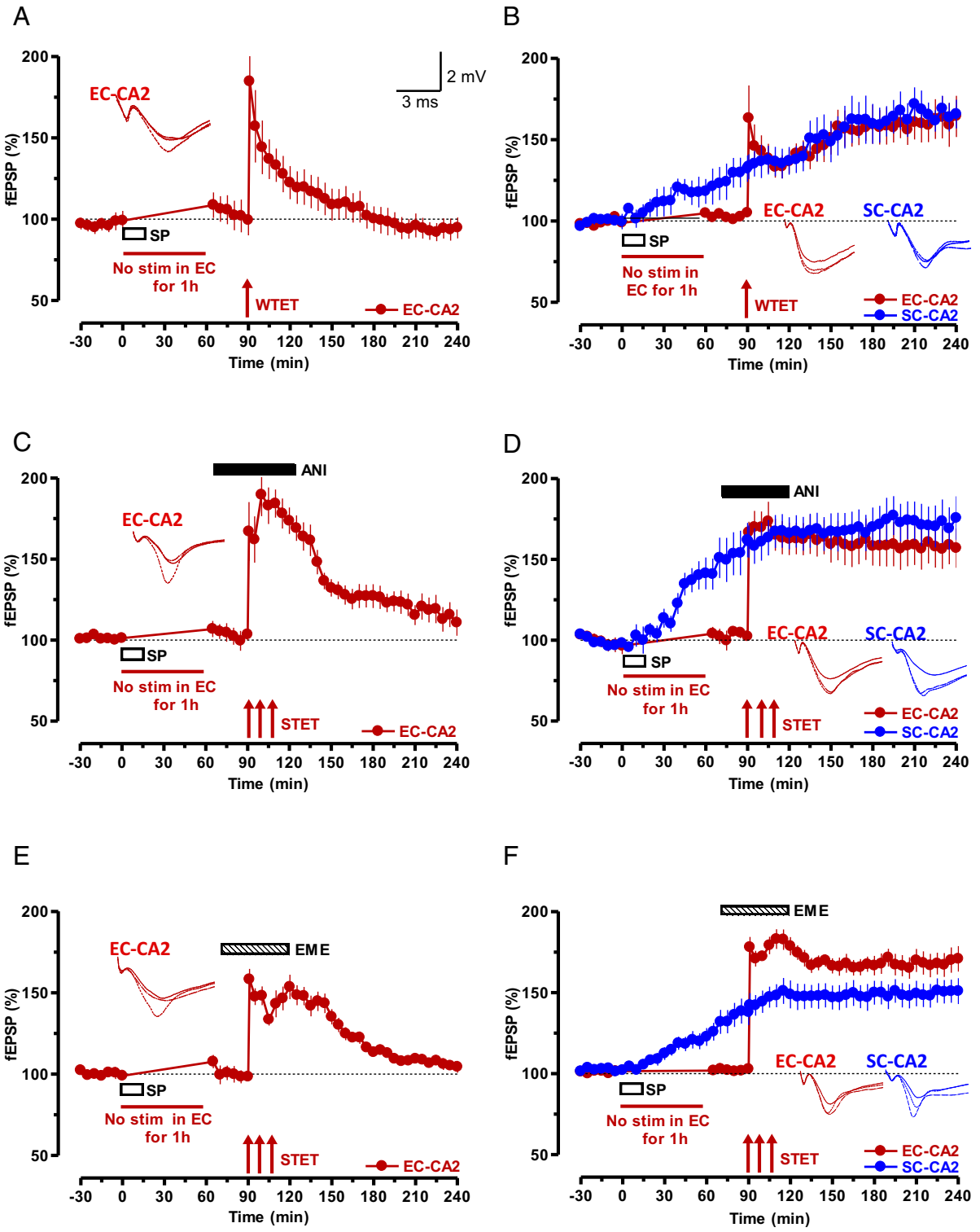

Fig. 3. SP initiates STC in SC-CA2 and EC-CA2 synapses. (*A*) Experiment showing a WTET-induced early LTP in EC-CA2 synaptic inputs. After recording a stable baseline of 30 min, SP was bath-applied for 15 min, during which time the baseline stimulation was suspended for 1 h ($n = 7$). After that, a baseline was recorded for another 30 min followed by WTET protocol. The potentiation decayed to baseline level within 180 min. (*B*) STC initiated by SP. Stimulation of EC-CA2 inputs was suspended during the application of SP and, at the 90th minute, WTET was delivered and the SC-CA2 fEPSPs were further recorded. Unlike in *A*, SP transformed the EC-CA2 fEPSP potentiation into an L-LTP ($n = 8$). (*C* and *E*) Experiments similar to *A* but with STET protocol was applied at the 90th minute in the presence of protein synthesis inhibitors ANI (25 μM; $n = 8$; *C*) or EME (20 μM; $n = 8$; *E*). The inhibitors were applied 20 min before STET (i.e., 10 min after resuming the baseline recording) and washed out 40 min after STET of the EC-CA2 input. (*D* and *F*) STC experiments in which L-LTP in the EC-CA2 inputs (red circles) was expressed in the presence of protein synthesis inhibitors ANI (25 μM; $n = 8$; *D*) or EME (20 μM; $n = 9$; *F*) as a result of the capture of previously SP-induced plasticity-relevant proteins. The experimental design was similar to that in *B* with the exception that, in this case, STET was applied to EC-CA2 inputs at the 90th minute. Representative fEPSP traces 15 min before (closed line), 95 min after (dotted line), and 180 min after (hatched line) SP application or WTET/STET are depicted. Calibration bars for fEPSP traces are 2 mV/3 ms. Arrows indicate the time points of WTET or STET.

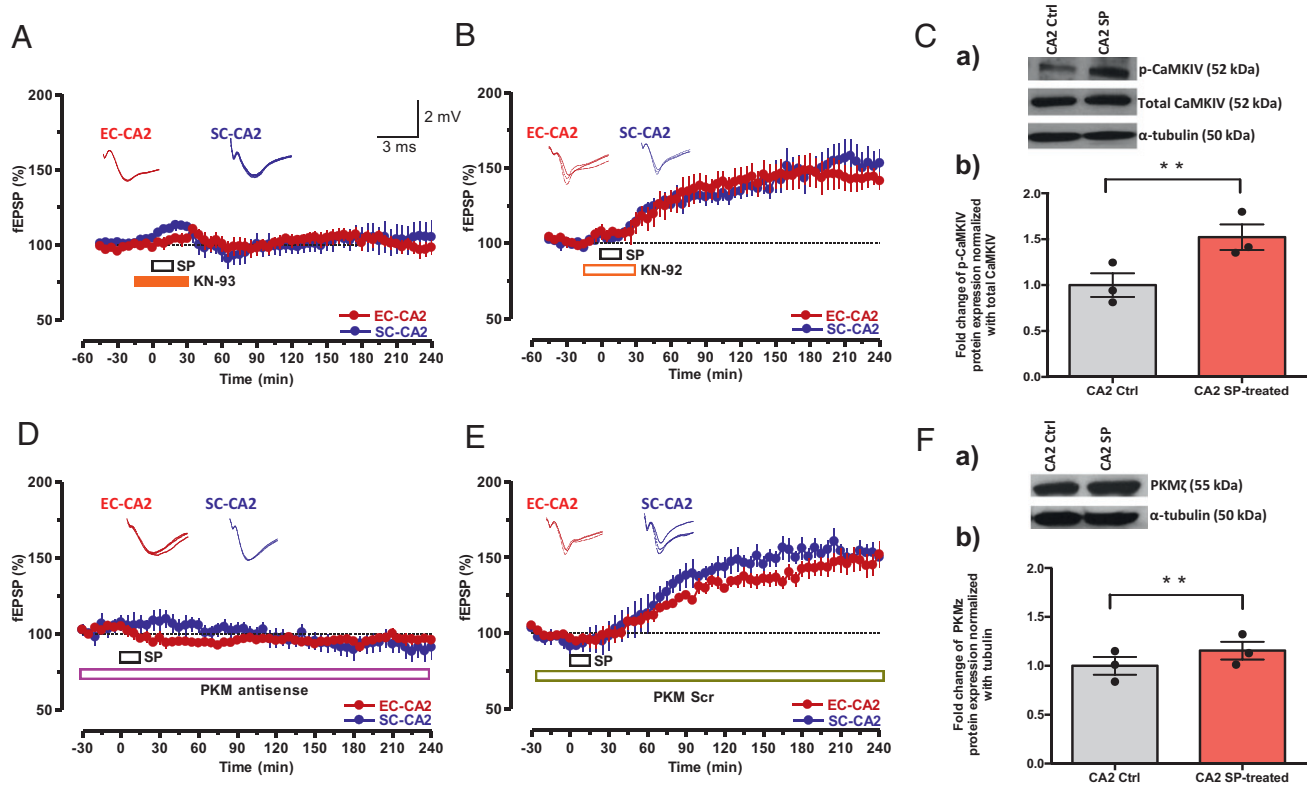

Fig. 4. PKMζ and CaMKIV are required for SP-induced plasticity and associativity in CA2 neurons. (*A*) The CaMKIV inhibitor KN-93 (10 μM) and SP were coapplied as indicated by horizontal bars in the graph (total of 45 min). The coapplication prevented the induction of SP-mediated potentiation in both synaptic inputs ($n = 8$). (*B*) Experimental design and drug application similar to that in *A* except that the nonactive drug KN-92 was used ($n = 8$). (*C*, *a* and *b*) Western blot analysis showed a significant increase of CaMKIV protein phosphorylation in the CA2 region after SP treatment compared with the respective control. The significant difference between the groups (CA2 control vs. CA2 SP-treated) is indicated by $^{**}P < 0.01$ (from three biological replicates). Individual data points of fold change are represented within the bar graphs. (*D*) Preincubation (1.5 h) and continuous application of PKMζ antisense oligodeoxynucleotides (20 μM) for as long as 240 min prevented SP-induced fEPSP potentiation ($n = 7$). (*E*) Experimental design similar to that in *D* except that a scrambled version of PKMζ antisense oligodeoxynucleotides was applied ($n = 7$). Representative fEPSP traces 15 min before (closed line), 60 min after (dotted line), and 180 min after (hatched line) SP application are depicted. Calibration bars for fEPSP traces are 2 mV/3 ms. (*F*, *a* and *b*) Western blot analysis of PKMζ protein expression also showed a significant up-regulation in CA2 region after SP treatment compared with control. The significant difference between the groups (CA2 control vs. CA2 SP-treated) is indicated by $^{**}P < 0.01$ (from three biological replicates). Individual data points of fold change are represented within the bar graphs.

An intriguing observation in the present study is the requirement of test stimulation for the expression of SP-induced potentiation. Interestingly, test stimulation was observed to be mandatory only during SP application, as experiments suspending test stimulation after establishing SP potentiation did not show interference with the STC process. In addition, test stimulation along with SP not only helps in the setting of synaptic tags but also quickly activates the synthesis of PRPs (here CaMKIV ad PKMζ) that are essential for the expression of plasticity in SC-CA2 and EC-CA2 synapses of area CA2. However, SP priming did not facilitate plasticity induction in SC-CA2 synapse, as STET failed to induce any visible plasticity, an interesting finding that needs further attention. Overall, the present findings on the role of test stimulation are consistent with our earlier observation that a synergistic role of dopaminergic D1/D5- as well as NMDA receptor-function is required for the maintenance of protein synthesis-dependent long-lasting plasticity in hippocampal area CA1 pyramidal neurons (26).

Chevaleyre and Siegelbaum (4) showed that cortical inputs can drive excitation in CA2 pyramidal neurons. The absence of excitation in CA2 from the intrahippocampal CA3 input makes it difficult to gauge the role of CA2 in the intrahippocampal network. Perhaps the feed-forward inhibition of CA3 inputs at CA2 serves to fine tune the information flow from CA3 to CA1. Nasrallah et al. (45) showed that LTD induction at inhibitory synapses (iLTD) in CA2 results in CA2 firing upon SC stimulation. Moreover, iLTD further increases the net excitatory drive of CA2 pyramidal neurons upon EC-LII stimulation and may serve to increase the cellular output at CA1 from the combined EC LII-dentate gyrus-CA3-CA2-CA1 and the more direct EC LII-CA2-CA1 loops. The inhibitory GABAergic transmission was shown to be a prerequisite for iLTD induction in CA2, as blocking of GABA receptors abolished this effect. A previous study (46) had already shown that the activation of delta opioid receptors (DORs) in the inhibitory interneurons is necessary to mediate iLTD in CA2 by decreasing solely the inhibitory transmission without altering excitatory transmission from CA3. Nasrallah et al. (45) showed that the increase in postsynaptic potential amplitude and action potential firing in CA2 upon HFS is also dependent on DOR activation, further confirming the role of DOR-mediated iLTD at inhibitory synapses onto CA2 in mediating potentiation in CA2. In general, a presynaptic inhibitory plasticity allows the net output of area CA2 to increase, i.e., a decrease in inhibition combined with no change in excitation leads to a net increase in CA2 pyramidal neuron output.

Unlike DORs, tachykinins are mostly known to cause a direct excitation of inhibitory interneurons and enhance the inhibitory inputs in the pyramidal neurons (14). Another study reported by Ogier et al. (47) shows that hippocampal GABAergic

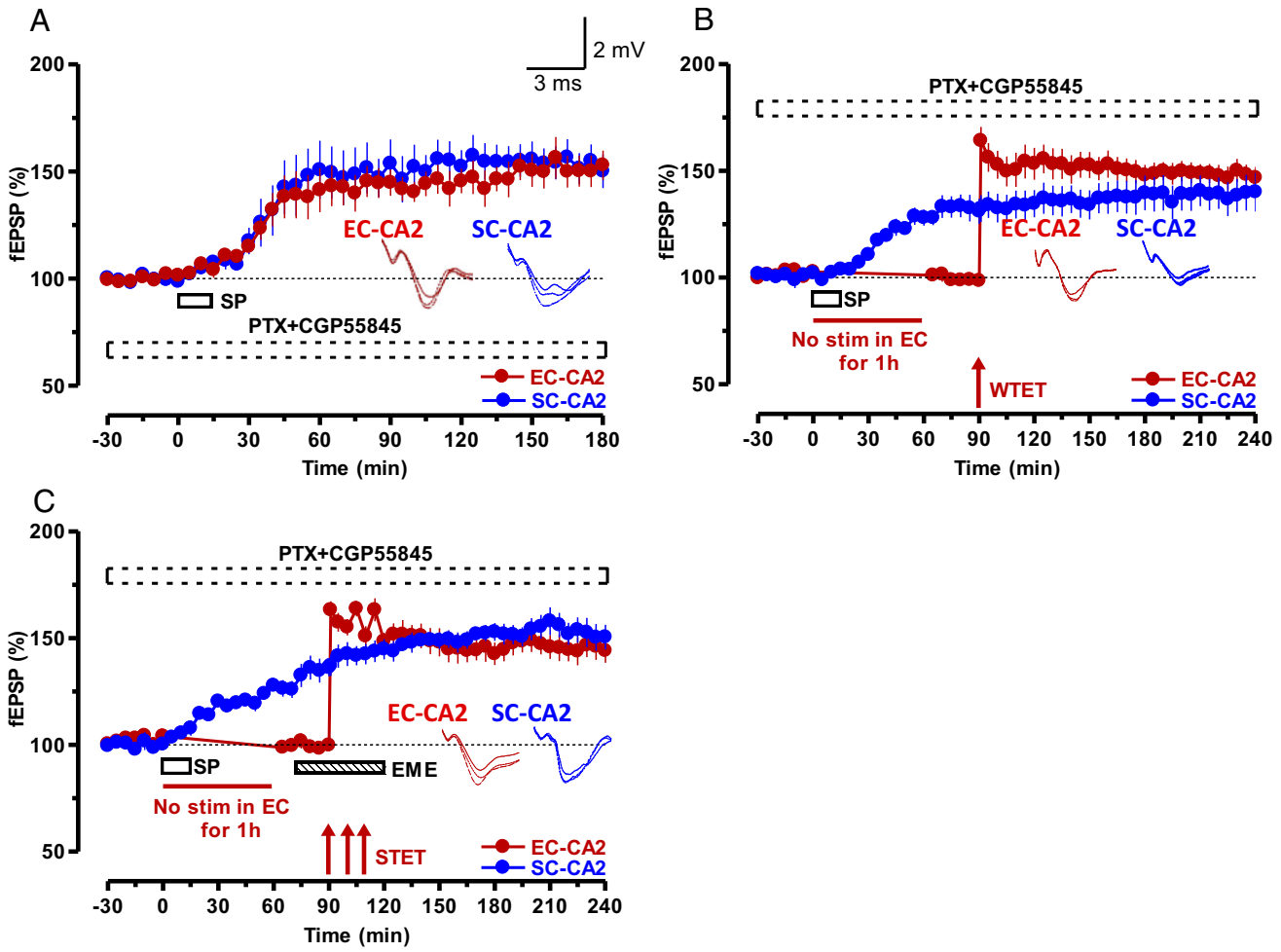

Fig. 5. SP-induced potentiation does not require $GABA_A$ or $GABA_B$ receptors. (*A*) Inhibitors of $GABA_A$ receptors, PTX (100 μM), or of $GABA_B$ receptors, CGP55845 (2 μM), were applied together during the entire incubation and recording period. SP was applied for 15 min. Potentiation was intact in SC-EC-CA2 synaptic inputs ($n = 6$). (*B*) Similar to the experiments in Fig. 3, STC experiments were carried out in the presence of the same $GABA_A$ and $GABA_B$ receptor inhibitors during the entire incubation and recording period. Even in the absence of GABAergic transmission, SP-mediated potentiation in SC-CA2 could transform the EC-CA2 fEPSP potentiation into an L-LTP ($n = 7$). (*C*) Experimental design was similar to that in Fig. 3*F*, but the experiment was carried out in the presence of $GABA_A$ and $GABA_B$ receptor inhibitors during the entire incubation and recording period ($n = 6$). STC was still observed even without GABAergic transmission. Representative fEPSP traces 15 min before (closed line), 95 min after (dotted line), and 180 min after (hatched line) SP application or WTET/STET are depicted. Calibration bars for fEPSP traces are 2 mV/3 ms.

interneurons possess tachykinin receptors (including NK1), which can engage in dual mechanisms by depressing or disinhibiting the activity of pyramidal neurons. These receptor-bearing interneurons can indirectly inhibit other interneurons as a result of the existence of interneuron-interneuron synapses, and this action causes a partial disinhibition of pyramidal neurons, leading to an indirect excitation of these cells. This possibility cannot be ruled out in the case of CA2, which is enriched with a dense population of interneurons. Although our studies did not show any difference in SP-mediated plasticity and STC processes in the presence of GABA inhibitors, it is not unlikely that the NK1 receptors in GABAergic interneurons would still be intact, even in the absence of GABAergic inhibition. Furthermore, according to Liu et al. (48), SP can increase the intracellular calcium concentration with a rapid enhancement in glutamate release. This in turn can activate more NMDA receptors and result in a large and long-lasting excitatory postsynaptic potential. Thus, an indirect inhibition and/or disinhibition of CA2 pyramidal neurons may play a critical role in maintaining SP-mediated plasticity in area CA2 during the inhibition of GABAergic transmission. Overall, the exact mechanism of SP-mediated neuromodulation in CA2 during the inhibition of GABAergic transmission is not very clear and needs further study.

The neuromodulator SP is proposed to be related to the transmission of pain information into the central nervous system (12). Neuromodulation of hippocampal area CA2 by SP possesses the potential to fine tune excitatory inputs onto the major hippocampal output CA1. As a result of the emerging evidence of different neuromodulatory substances capable of mediating plasticity in area CA2, it is becoming increasingly clear that the plasticity limiting properties of CA2 is functionally important, requiring the right signal at the right time. Social interactions and their resultant emotional repercussions could potentially influence one's day-to-day performance and ability to learn and remember. It will be interesting to understand if neuromodulatory inputs onto CA2 neurons during such social interactions and resultant stressors could, in turn, modulate information flow to CA1 and thereby the net output from CA1. Thus, we propose that modulation of synaptic inputs at CA2 could also have an impact on learning and memory functions other than social recognition memory.

As alterations to the CA2 region have been reported in neuropsychiatric illnesses such as schizophrenia, it is of clinical significance to understand whether changes in neuromodulatory inputs to CA2 are associated with the symptomatic deficits in day-to-day performance, learning and memory, social recognition memory, and social interactions. Response to novelty (49) and generation of hippocampal theta rhythm (50–53), two critical factors for long-term memory formation and maintenance, are influenced by SuM activity. Thus, given the crucial role of CA2 in social memory, it is highly likely that the SP-expressing afferents that uniquely innervate hippocampal area CA2 can potentially influence the consolidation of social memory and thereby influence social interactions. Our future study will address the specific role of SP in area CA2 and its influence on social memory.

Methods

All animal procedures were approved by guidelines from the institutional animal care and use committee of the National University of Singapore. More details about slice preparation, incubation, electrophysiology procedures, pharmacology, and molecular biology are provided in *SI Methods*.

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