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Diversity of neuropsin (KLK8)-dependent synaptic associativity in the hippocampal pyramidal neuron

Yasuyuki Ishikawa[1,2], Hideki Tamura[1,2] and Sadao Shiosaka[1,2]

[1] *Laboratory of Functional Neuroscience, Nara Institute of Science and Technology, 8916-5, Takayama, Ikoma, Nara 630-0192, Japan*
[2] *JST, CREST, 8916-5, Takayama, Ikoma, Nara 630-0192, Japan*

Non-technical summary Activity-dependent synaptic plasticity is widely accepted to provide a cellular basis for learning and memory. Synaptic associativity could be involved in activity-dependent synaptic plasticity, because it distinguishes between local mechanisms of synaptic tags and cell-wide mechanisms that are responsible for the synthesis of plasticity-related proteins. We report that a plasticity-related serine protease, neuropsin, is involved in the tag-setting process during long-term potentiation (LTP) at basal and apical dendritic inputs. Neuropsin is involved in synaptic associativity during LTP at apical dendritic inputs via integrin β1 and CaMKII signalling. Thus, neuropsin is a candidate molecule for LTP-specific tag-setting and could regulate the transformation of early to late LTP during synaptic associativity. These findings may provide the understanding of the regulation of synaptic associativity as complex information-processing systems.

Abstract Hippocampal early (E-) long-term potentiation (LTP) and long-term depression (LTD) elicited by a weak stimulus normally fades within 90 min. Late (L-) LTP and LTD elicited by strong stimuli continue for >180 min and require new protein synthesis to persist. If a strong tetanus is applied once to synaptic inputs, even a weak tetanus applied to another synaptic input can evoke persistent LTP. A synaptic tag is hypothesized to enable the capture of newly synthesized synaptic molecules. This process, referred to as synaptic tagging, is found between not only the same processes (i.e. E- and L-LTP; E- and L-LTD) but also between different processes (i.e. E-LTP and L-LTD; E-LTD and L-LTP) induced at two independent synaptic inputs (cross-tagging). However, the mechanisms of synaptic tag setting remain unclear. In our previous study, we found that synaptic associativity in the hippocampal Schaffer collateral pathway depended on neuropsin (kallikrein-related peptidase 8 or KLK8), a plasticity-related extracellular protease. In the present study, we investigated how neuropsin participates in synaptic tagging and cross-tagging. We report that neuropsin is involved in synaptic tagging during LTP at basal and apical dendritic inputs. Moreover, neuropsin is involved in synaptic tagging and cross-tagging during LTP at apical dendritic inputs via integrin β1 and calcium/calmodulin-dependent protein kinase II signalling. Thus, neuropsin is a candidate molecule for the LTP-specific tag setting and regulates the transformation of E- to L-LTP during both synaptic tagging and cross-tagging.

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Corresponding author Y. Ishikawa: Laboratory of Functional Neuroscience, Nara Institute of Science and Technology, 8916-5, Takayama, Ikoma, Nara 630-0192, Japan. Email: yishikaw@bs.naist.jp

Abbreviations CaMKII, calcium/calmodulin-dependent protein kinase II; ERK, extracellular signal-regulated kinase; fEPSP, field EPSP; HFS, high frequency stimulation; KO, knock-out; LFS, low frequency stimulation; LTD, long-term depression (E-, early; L-, late); LTP, long-term potentiation (E-, early; L-, late); MMP, matrix metalloprotease; PRP, plasticity-related protein; rmNP, recombinant mouse (pro)neuropsin; WT, wild-type.

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Introduction

Hippocampal CA1 pyramidal neurons receive a variety of inputs from the circuitry and physiology of other brain areas thought to be involved in learning and memory. These synaptic inputs may be integrated and associated, and thereby engaged in the processes of association memory. Frey & Morris (1997) first reported synaptic late associativity between early long-term potentiation (E-LTP) and late (L-) LTP in hippocampal CA1 apical dendrites when weak and strong stimuli were delivered to two separate synaptic inputs. They found that LTP induced by weak stimulation could not persist until the delivery of new plasticity-related proteins (PRPs) that are provided by signals from other L-LTP-induced synaptic inputs. Therefore, they hypothesized that weak synaptic input stimulation produces only a hypothetical synaptic mark, or tag (Frey & Morris, 1997, 1998; Martin & Kosik, 2002; Morris, 2006; Barco *et al.* 2008; Frey & Frey, 2008).

Several types of late associativity have been reported not only between the same processes (synaptic tagging; i.e. E- and L-LTP, E- and L-long-term depression; LTD), but also between different processes (cross-tagging; i.e. E-LTP and L-LTD, E-LTD and L-LTP), when each is induced by two independent synaptic inputs. Such associativities were also found between different dendritic compartments (cross-compartmentalization) under unconventional conditions (Sajikumar & Frey, 2004; Alarcon *et al.* 2006; Sajikumar *et al.* 2007). Collectively, hippocampal late associativities may be complex information-processing systems that are hypothesized to act as a hub or centre for integration in novel, recalled, and often emotional memories.

We have reported that neuropsin modulates Schaffer-collateral E-LTP in a dose-dependent manner through the cleaving of cell adhesion or matrix proteins (Shimizu *et al.* 1998; Komai *et al.* 2000; Matsumoto-Miyai *et al.* 2003; Tamura *et al.* 2006). Neuropsin is activated during LTP in an NMDA receptor-dependent manner. Activation of neuropsin occurs by cleavage of a 4-amino acid peptide (QGSK) after a signal is received from downstream of the NMDA receptor. However, the mechanism of activation of neuropsin still remains unclear. Previously, we revealed the existence of neuropsin-dependent late associativity between E-LTP and L-LTP (Ishikawa *et al.* 2008). However, other questions remain to be determined, including what other factors are involved in neuropsin-dependent associativity and the role of neuropsin signalling in forms of neuropsin-dependent associativity other than late associativity. To clarify these mechanisms, in the present study we investigated the role of neuropsin in synaptic tagging and cross-tagging in hippocampal slices.

Methods

Hippocampal slice preparation

All experiments were conducted with male C57BL/6J mice and neuropsin-deficient mice (age, 6–8 weeks). Mice were maintained according to the guidelines of the Nara Institute of Science and Technology, and the study was approved by the institutional Animal Care and Use Committee. Mice were anaesthetized I.P. with urethane (1.25 g (kg body weight)$^{-1}$: 10% ethyl carbamate (12.5 ml (kg body weight)$^{-1}$) at room temperature). The toe or tail pinch reflexes were used to assess the level of anaesthesia and these were abolished at surgical anaesthesia. Anaesthetized animals were transcardially perfused with ice-cold artificial CSF (ACSF) for blood removal and cooling of the brain. They were decapitated, and brains were removed and immersed in ice-cold (4°C) ACSF bubbled with a mixture of 95% O_2 and 5% CO_2. ACSF consisted of 125 mM NaCl, 2.6 mM KCl, 1.3 mM $MgSO_4.7H_2O$, 1.24 mM KH_2PO_4, 26 mM $NaHCO_3$, 2.4 mM $CaCl_2$, and 10 mM D-glucose. The hippocampi were dissected free, and transverse slices (400 μm thickness) were cut on a slicer (LinearSlicer Pro7; Dosaka, Kyoto, Japan). Slices were allowed to recover at 30°C for 30 min and were maintained at room temperature for at least 120 min before experiments.

Electrophysiology

Extracellular field EPSPs (fEPSPs) were recorded in the stratum radiatum and/or the stratum oriens of area CA1 with a glass microelectrode (Narishige, Tokyo, Japan) filled with ACSF (electrical resistance, 2–4 MΩ). Extracellular stimulation of the Schaffer-collateral pathway was performed with two nickel–chromium (Unique Medical, Tokyo, Japan) bipolar stimulating electrodes (diameter, 40 μm) placed on a recording electrode in the stratum radiatum and/or the stratum oriens. Evoked fEPSPs were amplified (ER-1 amplifier; Cygnus, Delaware Water Gap, PA, USA), digitized (DigiData 1200 Interface; Molecular Devices, Palo Alto, CA, USA), and analysed using the LTP program (Anderson & Collingridge, 2001). The test stimulus intensity was adjusted to produce a 'baseline' fEPSP that was 40% of the maximal evoked fEPSP slope (SIU-91 stimulus isolation unit; Cygnus). Test stimuli were delivered once per minute (0.2 ms stimulus duration) to Schaffer collaterals with a 30 s separation between stimulations through the two electrodes (stimulating electrodes S0 and S1). To ensure that fEPSPs evoked through each stimulating electrode resulted from the activation of two independent synaptic pathways, we positioned the electrodes so that no paired-pulse facilitation (PPF) was evident after sequential activation at

S0 and S1. The interpathway PPF was assessed at various time intervals (50 and 500 ms) before baseline acquisition.

LTP was elicited by delivering a phase-specific potentiation protocol. Weak high frequency stimulation (weak-HFS; 1 s duration at 100 Hz) induced E-LTP, and strong high frequency stimulation (strong-HFS; four episodes of the train composed of 100 pulses (at 100 Hz), inter-episode interval, 5 min) induced L-LTP. LTD was also elicited by delivering a phase-specific potentiation protocol. Weak low frequency stimulation (weak-LFS; 15 min duration at 1 Hz) induced E-LTD; strong low frequency stimulation (strong-LFS; 900 bursts; a single burst consisted of three pulses, constant current stimuli at 20 Hz with a 1 s interburst interval and 0.2 ms stimulus duration) induced L-LTD. Basal transmission (input–output and PPF) did not significantly differ between wild-type (WT) and neuropsin knock-out (KO) mice at apical and basal dendrites (data not shown).

Drugs

2-[*N*-(2-Hydroxyethyl)]-*N*-(4-methoxybenzenesulfonyl))-amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (KN93; Calbiochem, San Diego, CA, USA) was added to ACSF at a final concentration of 5 μM. 1,4-Diamino-2,3-dicyano-1,4-bis [2-amino-phenylthio]butadiene (U0126; Promega, Madison, WI, USA) was added to ACSF at a final concentration of 10 μM. 4-Amino-5-(4-chloro-phenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2, Calbiochem) was added to ACSF at a final concentration of 10 μM. Integrin β1 antibody (1987z; Chemicon, Billerica, MA, USA; and Ha 2/5; BD Pharmingen, Franklin Lakes, NJ, USA) was added to ACSF at a final concentration of 100 μg ml^{-1}. KN93, U0126 and PP2 were bath applied. The final concentration of applied DMSO was 0.01%. At this concentration, baseline fEPSP slopes were not significantly affected (see Supplemental Fig. 1, available online only). Integrin β1 antibody was applied locally using an injection pump (0.5 μl min^{-1}). The pre-activated recombinant mouse (pro)neuropsin (rmNP) (0.3 mU ml^{-1}) was bath applied (Ishikawa *et al.* 2008).

Statistical analysis

The initial fEPSP slope was measured and expressed as a percentage of the averaged baseline. The latter was obtained by averaging 30 min of fEPSPs measured during baseline acquisition. Data are plotted as the mean ± SEM. Data sets were analysed with the Mann–Whitney *U* test.

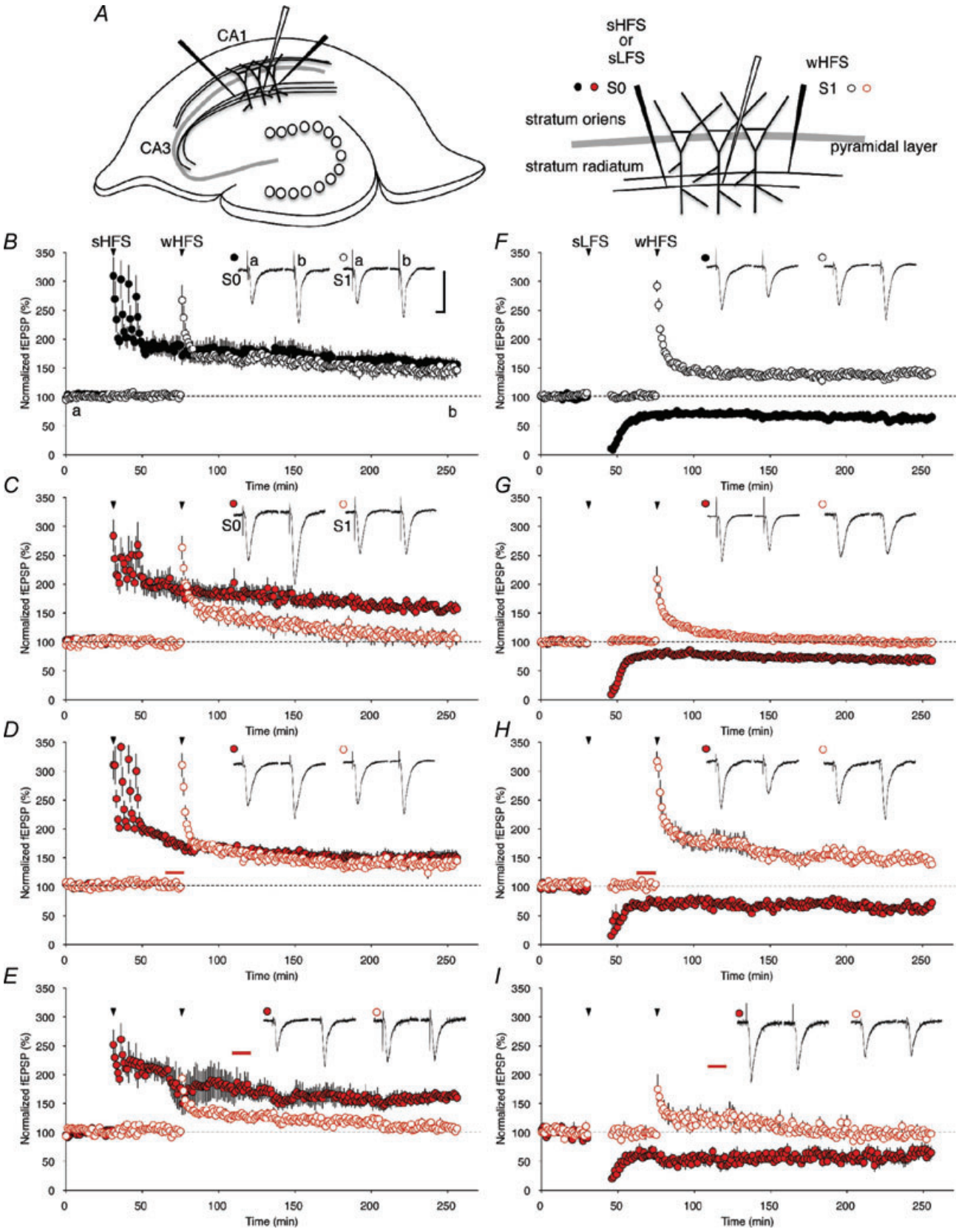
Results

Neuropsin-dependent synaptic associativity in CA1 pyramidal neurons

The neuropsin-dependent late associativity observed in our previous study was confirmed using weak-HFS after strong-HFS protocols in hippocampal slices from wild-type (WT) and neuropsin knock-out (KO) mice (Ishikawa *et al.* 2008). Weak-HFS induced synaptic tagging in WT hippocampal slices but not in KO hippocampal slices (180 min after tetanus: WT, 147 ± 12 at S1, $n = 7$, Fig. 1*B*; KO, 107 ± 9 at S1, $n = 6$, Fig. 1*C*; WT *vs.* KO, Mann–Whitney *U* test, $P < 0.05$). This impairment of late associativity in KO hippocampal slices was reversed by a brief (10 min) bath application of rmNP (180 min after tetanus: KO + rmNP, 141 ± 5 at S1, $n = 6$, Fig. 1*D*; KO *vs.* KO + rmNP, Mann–Whitney *U* test, $P < 0.05$) (Ishikawa *et al.* 2008). Thus, a form of synaptic tagging occurs in the apical dendrite of CA1 pyramidal neurons, of which neuropsin is an essential extracellular modulator.

We next addressed whether a neuropsin-dependent form of late associativity that occurs only between two synaptic inputs induced the same process. To elucidate mechanisms underlying cross-tagging, we attempted to induce neuropsin-dependent cross-tagging between E-LTP and L-LTD. As shown in Fig. 1*F*, when L-LTD was induced at S0 followed by E-LTP at S1, transformation of E-LTP into persistent L-LTP (in S1) was observed in WT hippocampal slices (cross-tagging; 180 min after tetanus: 141 ± 2 at S1, $n = 6$), as is the case between E- and L-LTP. This type of late association was also clearly neuropsin dependent because L-LTD at S0 followed by induction of E-LTP at S1 did not transform E-LTP into persistent L-LTP at S1 in KO hippocampal slices (180 min after tetanus: 99 ± 4 at S1, $n = 7$, Fig. 1*G*; WT *vs.* KO, Mann–Whitney *U* test, $P < 0.005$). Moreover, this impairment was rescued by a brief bath application of rmNP (180 min after tetanus: KO + rmNP, 147 ± 4 at S1, $n = 6$, Fig. 1*H*; KO *vs.* KO + rmNP, Mann–Whitney *U* test, $P < 0.005$). Next, we infused rmNP at 30 min after the weak-HFS or before weak-HFS to investigate the time window of the neuropsin effect in KO hippocampal slices. The impairments were not rescued by a brief bath application of rmNP at 30 min after the weak-HFS (synaptic tagging; 180 min after tetanus: KO + rmNP after 30 min, 108 ± 3 at S1, $n = 7$, Fig. 1*E*; cross-tagging; 180 min after tetanus: KO + rmNP after 30 min, 97 ± 2 at S1, $n = 5$, Fig. 1*I*). Therefore, these findings suggest that neuropsin is involved in an extracellular LTP tag setting process during the induction of E-LTP.

Our next question was whether E-LTD induced a neuropsin-dependent synaptic tag. Analogous to the experiments on E-LTP, we investigated whether L-LTP or L-LTD at S0 followed by the induction of E-LTD at S1 gave rise to similar late associativity. Figure 2 shows



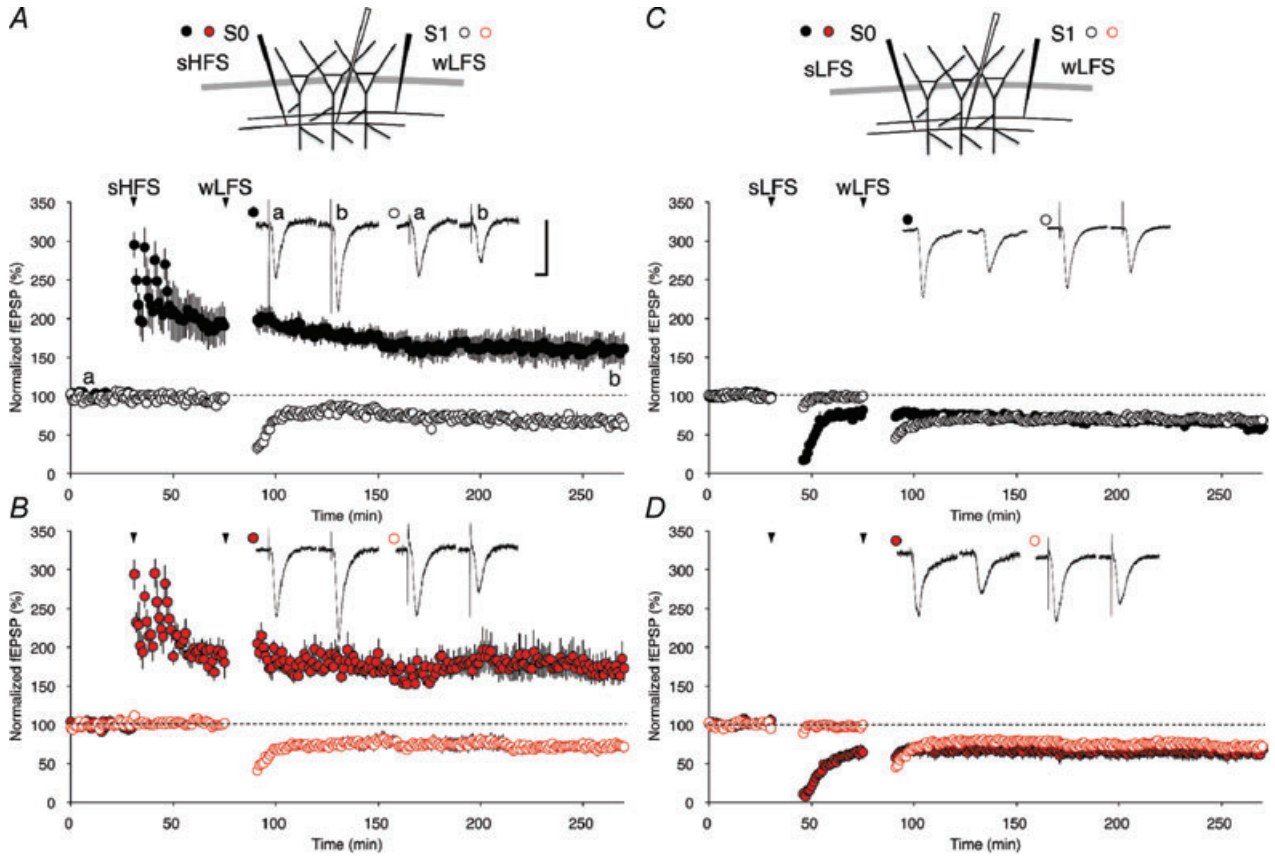


Figure 2. Neuropsin is not involved in E-LTD-related synaptic associativity
Weak-LFS induced late associativity in WT and neuropsin-KO hippocampal slices from mice. E-LTD-related late associativity does not require neuropsin. *A,* schematic diagram of a hippocampal slice showing positions of two stimulating electrodes (S0 and S1) and a single recording electrode placed in the stratum radiatum of area CA1. Weak-LFS at S1 after strong-HFS at S0 resulted in stable L-LTD at S1 in WT hippocampal slices. *B,* in KO hippocampal slices, fEPSP values also demonstrated persistent LTD at S1 180 min post-weak-LFS after strong-HFS at S0. *C,* schematic diagram of a hippocampal slice showing positions of two stimulating electrodes (S0 and S1) and a single recording electrode placed in the stratum radiatum of area CA1. Weak-LFS at S1 after strong-LFS at S0 resulted in stable L-LTD at S1 in WT hippocampal slices. *D,* in KO hippocampal slices, fEPSP values also demonstrated persistent LTD at S1 180 min post-weak-LFS after strong-LFS at S0. sHFS and wHFS indicate strong-HFS and weak-HFS, respectively. sLFS and wLFS indicate strong-LFS and weak-LFS, respectively. Error bars indicate SEM. Inset traces show sample fEPSPs recorded during the baseline period (a) and at 180 min after stimulation (b) at S1. Calibration: 1 mV, 10 ms.

Figure 1. Neuropsin-dependent synaptic associativity in CA1 pyramidal neurons is E-LTP specific
Weak-HFS induced late associativity in hippocampal slices from WT mice but not KO mice. *A*, schematic diagram of a hippocampal slice showing the positions of two stimulating electrodes (S0 and S1) and a single recording electrode placed in the stratum radiatum of area CA1. *B,* weak-HFS at S1 (open circles) after strong-HFS at S0 (filled circles) resulted in stable L-LTP at S1 in WT hippocampal slices. *C,* by contrast, in KO hippocampal slices, fEPSP values were impaired at S1 (red open circles) 180 min post-tetanus after strong-HFS at S0 (red filled circles). *D,* rmNP administration to KO slices reversed the induction of L-LTP at weakly stimulated S1. *F,* weak-HFS at S1 after strong-LFS at S0 resulted in stable L-LTP at S1 in WT hippocampal slices. *G,* by contrast, in KO hippocampal slices, fEPSP values were impaired at S1 180 min post-tetanus after strong-LFS at S0. *H,* rmNP administration in KO hippocampal slices reversed the induction of L-LTP at weakly stimulated S1. *E* and *I,* rmNP was infused into slices at 30 min after weak-HFS as well as before weak-HFS to investigate the time window of the neuropsin effect in KO hippocampal slices. The impairments were not rescued by a brief bath application of rmNP at 30 min after weak-HFS at S1 after strong-HFS (*E*) or strong-LFS (*I*) at S0. Red bar indicates rmNP administration. sHFS and wHFS indicate strong-HFS and weak-HFS, respectively. sLFS indicates strong-LFS. Error bars indicate SEM. Inset traces show sample fEPSPs recorded during the baseline period (a) and at 180 min after stimulation (b) at S1. Calibration: 1 mV, 10 ms.

that weak-LFS for E-LTD at S1 after strong-HFS for L-LTP at S0 transformed E-LTD into persistent L-LTD at S1 in WT hippocampal slices (180 min after stimulus: 66 ± 6 at S1, $n = 5$, Fig. 2*A*). Unlike the case of E-LTP described above (Fig. 1*C* and *G*), no defect was seen in KO hippocampal slices (180 min after stimulus: 73 ± 7 at S1, $n = 6$, Fig. 2*B*; WT *vs.* KO, Mann–Whitney *U* test, not significant). In addition, weak-LFS for E-LTD at S1 after strong-LFS for L-LTD at S0 also transformed E-LTD into persistent L-LTD at S1 in WT (180 min after stimulus: 68 ± 2 at S1, $n = 7$, Fig. 2*C*) and KO hippocampal slices (180 min after stimulus: 72 ± 2 at S1, $n = 6$, Fig. 2*D*; WT *vs.* KO, Mann–Whitney *U* test, not significant). These experiments indicate that neuropsin is not involved in the E-LTD tag-setting process.

Neuropsin-dependent synaptic associativity was restricted within local dendritic compartments

Synaptic associativity between two independent synaptic inputs has been reported not only in apical dendrites, but also in basal dendrites (Alarcon *et al.* 2006; Sajikumar *et al.* 2007). Therefore, we next examined the associativity between two independent synaptic inputs localized in the basal dendritic compartment. Similar to the apical compartment, neuropsin-dependent synaptic tagging and cross-tagging also occurred between two synaptic inputs in the basal dendritic compartment (synaptic tagging; 180 min after tetanus: WT, 132 ± 5 at S1, $n = 6$, Fig. 3*A*; KO, 97 ± 4 at S1, $n = 6$, Fig. 3*B*; WT *vs.* KO, Mann–Whitney *U* test, $P < 0.005$; cross-tagging; 180 min after tetanus: WT, 147 ± 7 at S1, $n = 4$, Fig. 3*D*; KO, 107 ± 8 at S1, $n = 7$, Fig. 3*E*; WT *vs.* KO, Mann–Whitney *U* test, $P < 0.05$). Moreover, this impairment was rescued by a brief bath application of rmNP (synaptic tagging; 180 min after tetanus: KO + rmNP, 142 ± 5 at S1, $n = 6$, Fig. 3*C*; KO *vs.* KO + rmNP, Mann–Whitney *U* test, $P < 0.01$; cross-tagging; 180 min after tetanus: KO + rmNP, 157 ± 10 at S1, $n = 6$, Fig. 3*F*; KO *vs.* KO + rmNP, Mann–Whitney *U* test, $P < 0.01$).

We further analysed whether neuropsin-dependent associativity across two independent synaptic inputs was localized to the apical and basal dendritic compartments. Separate synaptic inputs into apical and basal dendritic compartments, with weak-HFS at S1 after strong-HFS at S0, were applied to investigate cross-compartmentalization. The position of stimulating electrodes were changed of S0 and S1 for alternate experiments. In WT hippocampal slices and neuropsin-KO hippocampal slices, E-LTP at S1 induced by weak-HFS could not be transformed into persistent L-LTP across dendritic compartments (180 min after tetanus: WT basal, 105 ± 8 at S1, $n = 5$, Fig. 4*A*; WT apical, 100 ± 4 at S1, $n = 6$, Fig. 4*C*; KO basal, 96 ± 5 at S1, $n = 6$, Fig. 4*B*; KO apical: 102 ± 4 at S1, $n = 5$, Fig. 4*D*).

Slightly stronger stimulation (moderate-HFS), which was originally performed by Alarcon *et al.* (2006), has the potential to induce cross-compartmentalization (Alarcon *et al.* 2006) and induced neuropsin-independent synaptic tagging at apical dendrites (Ishikawa *et al.* 2008). Using this protocol, E-LTP at S1 could be transformed into L-LTP across two dendritic compartments not only in WT, but also in KO hippocampal slices (data not shown). Therefore, neuropsin is irrelevant in cross-compartmentalization, and the neuropsin-dependent form of associativity may be restricted to a local (presumably single) compartment of apical or basal dendrites.

Weak before strong protocol: neuropsin is required for synaptic tagging and cross-tagging

In addition to the above 'weak after strong' protocol, we also used a 'weak before strong' protocol to test the neuropsin-dependent synaptic tagging and cross-tagging, which has also been widely used in testing synaptic tagging. Similar to the weak after strong protocol, neuropsin-dependent synaptic tagging and cross-tagging also occurred between two synaptic inputs in the apical dendritic compartment (weak-HFS before strong-HFS/LFS, 225 min after weak-HFS: WT, synaptic tagging, 137 ± 12 at S1, $n = 6$, Fig. 5*A*; cross-tagging, 131 ± 4 at S1, $n = 5$, Fig. 5*D*; KO, synaptic tagging, 107 ± 5 at S1, $n = 8$, Fig. 5*B*; cross-tagging, 90 ± 6 at S1, $n = 6$, Fig. 5*E*; KO + rmNP, synaptic tagging, 137 ± 6 at S1, $n = 5$, Fig. 5*C*; cross-tagging, 146 ± 7 at S1, $n = 8$, Fig. 5*F*).

The role of integrin β1 and calcium/calmodulin-dependent protein kinase II (CaMKII) in neuropsin-dependent late associativity

Previously, we demonstrated a possible relationship between integrin signalling and neuropsin-dependent associativity using a wide-spectrum integrin-antagonist peptide, GRGDSP, and we and other investigators suggested that neuropsin and integrin may be particularly important in early LTP signalling, with functional similarity. Integrins are heterodimers of α- and β-subunits. Currently, 19 different α-subunits and 8 β-subunits are known in vertebrates, and over 20 different α/β heterodimers have been described. The integrins α-3, -5 and -8 are expressed in the hippocampus and are involved in synaptic plasticity and memory (Staubli *et al.* 1998; Chan *et al.* 2003, 2006; Huang *et al.* 2006; Tamura *et al.* 2006). In addition, fibronectin and L1cam, both of which are integrin ligands, are potential sub-

strates of neuropsin protease activity (Shimizu *et al.* 1998; Matsumoto-Miyai *et al.* 2003). We thought that integrin β1 would be a good candidate for studying integrin function in the hippocampus because it is the only known subunit partner for several integrin α-subunits, including α-3, -5 and -8. Therefore, we further analysed the blockade of integrin signalling using an antagonist: a neutralizing antibody directed against integrin β1 or inhibitors of CaMKII, extracellular signal-regulated kinase (ERK), and Src-family tyrosine kinase (Kramar

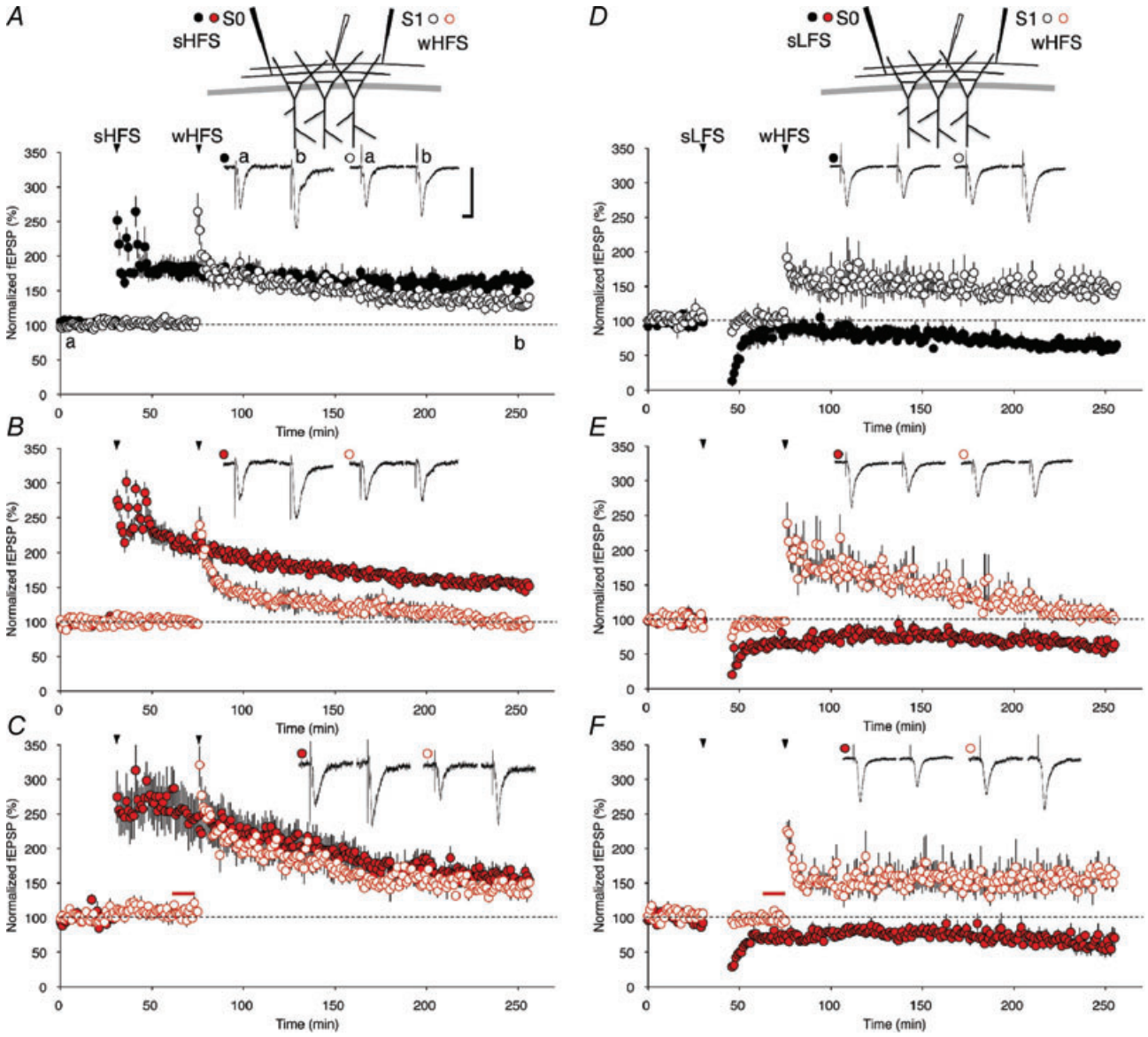


Figure 3. Neuropsin-dependent synaptic tagging and cross-tagging in the stratum oriens of area CA1
A, schematic diagram of a hippocampal slice showing positions of two stimulating electrodes (S0 and S1) and a single recording electrode placed in the stratum oriens of area CA1. Weak-HFS at S1 after strong-HFS at S0 resulted in stable L-LTP at S1 in basal dendrites of WT hippocampal slices. *B,* by contrast, in KO hippocampal slices, fEPSP values were impaired at S1 180 min post-tetanus after strong-HFS at S0 in basal dendrites. *C,* rmNP administration to KO slices reversed the induction of L-LTP at weakly stimulated S1. *D,* schematic diagram of a hippocampal slice showing the positions of two stimulating electrodes (S0 and S1) and a single recording electrode placed in the stratum oriens of area CA1. Weak-HFS at S1 after strong-LFS at S0 resulted in stable L-LTP at S1 in WT hippocampal slices. *E,* by contrast, in KO hippocampal slices, fEPSP values were impaired at S1 180 min post-tetanus after strong-LFS at S0. *F,* rmNP administration in KO hippocampal slices reversed the induction of L-LTP at weakly stimulated S1. Red bar indicates rmNP administration. sHFS and wHFS indicate strong-HFS and weak-HFS, respectively. sLFS indicates strong-LFS. Error bars indicate SEM. Inset traces show sample fEPSPs recorded during the baseline period (a) and at 180 min after stimulation (b) at S1. Calibration: 1 mV, 10 ms.

et al. 2003; Shi & Ethell, 2006; Watson *et al.* 2007). Using neuropsin-KO hippocampal slices supplemented with rmNP, late associativity caused by weak-HFS after strong-HFS or strong-LFS was clearly eliminated by the application of integrin antibody, but not control Ig (integrin β1 neutralizing antibody, 180 min after tetanus: synaptic tagging, 105 $\pm$ 3 at S1, $n = 7$, Fig. 6*A*; cross-tagging, 100 $\pm$ 6 at S1, $n = 8$, Fig. 6*E*; control Ig, 180 min after tetanus: synaptic tagging, 147 $\pm$ 10 at S1, $n = 4$, Supplemental Fig. 2*A*; cross-tagging, 136 $\pm$ 9 at S1, $n = 4$, Supplemental Fig. 2*B*).

The same experiments were performed using WT hippocampal slices to exclude any artificial effects introduced by rmNP. Local application of the integrin β1 antibody with weak-HFS at S1 completely eliminated synaptic tagging by weak-HFS after strong-HFS and cross-tagging by weak-HFS after strong-LFS on WT hippocampal slices (integrin β1 neutralizing antibody, 180 min after tetanus: synaptic tagging, 111 $\pm$ 3 at S1, $n = 8$, Fig. 7*A*; cross-tagging, 100 $\pm$ 6 at S1, $n = 8$, Fig. 7*E*). These results imply that integrin signalling may be a downstream component of neuropsin signalling in neuropsin-dependent association, and thus, neuropsin and integrin may share their signalling processes in associativity.

Further neuropsin-dependent associated signalling was examined by bath application of KN93, U0126 and PP2 (inhibitors of CaMKII, ERK, and Src-family tyrosine kinase, respectively) to neuropsin-KO hippocampal slices supplemented with rmNP. Use of KO hippocampal slices with acute extracellular application of rmNP in the following experiments aimed to examine how neuropsin participates in a series of kinase activations within a limited time window. Weak-HFS after strong-HFS/LFS protocols were used in this study to investigate changes in late associativity associated

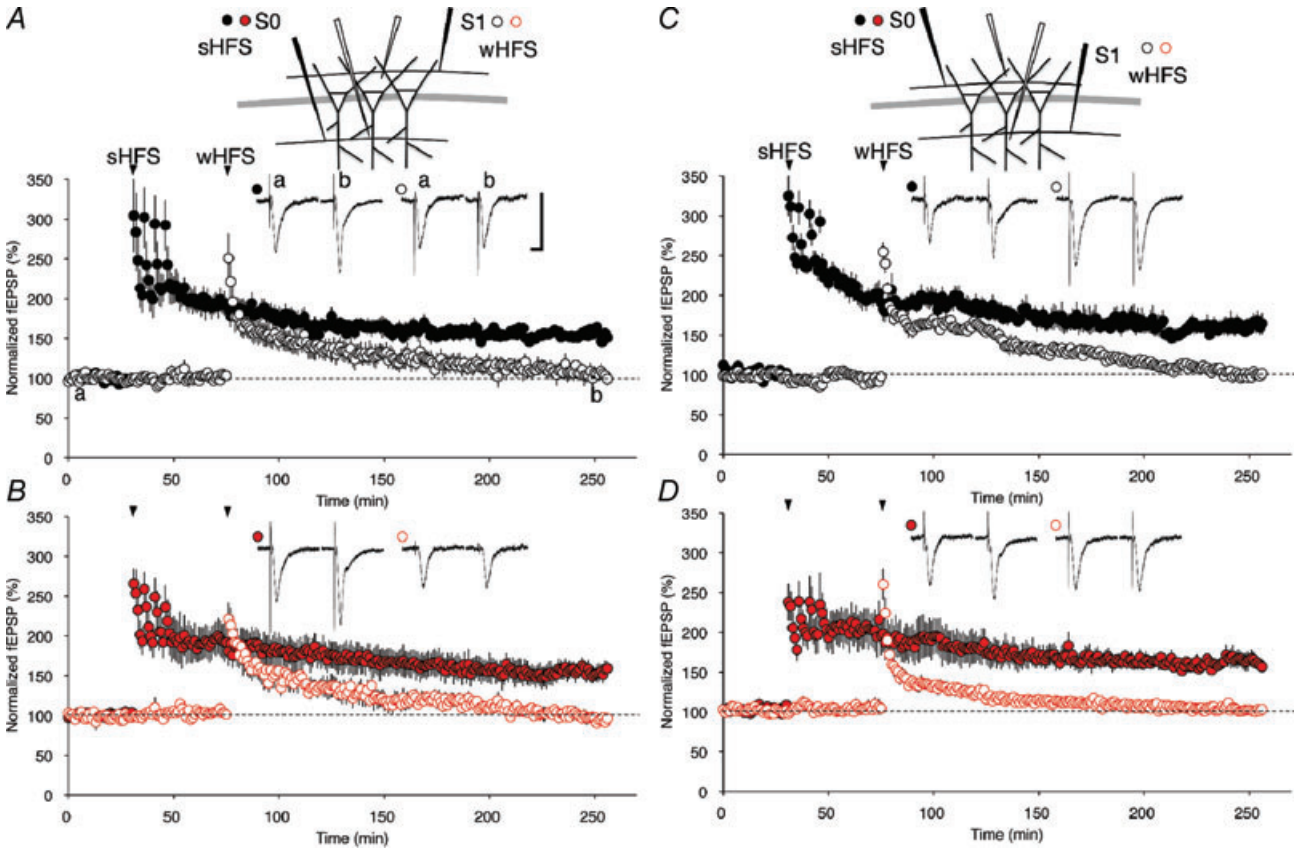


Figure 4. Neuropsin-dependent synaptic associativity is restricted to local dendritic compartments
A and *B,* schematic diagram of a hippocampal slice showing positions of two stimulating electrodes (S0, stratum radiatum; S1, stratum oriens) and two recording electrodes placed in the stratum radiatum and stratum oriens of area CA1. After L-LTP is evoked on apical dendrites at S0, weak-HFS fails to elicit persistent LTP in basal dendrites at S1 in WT (*A*) and KO (*B*) hippocampal slices. *C* and *D,* schematic diagram of a hippocampal slice showing positions of two stimulating electrodes (S0, stratum oriens; S1, stratum radiatum) and two recording electrodes placed in the stratum radiatum and stratum oriens of area CA1. After L-LTP is evoked on basal dendrites at S0, weak-HFS fails to elicit persistent LTP in apical dendrites at S1 in WT (*C*) and KO (*D*) hippocampal slices. sHFS and wHFS indicate strong-HFS and weak-HFS, respectively. Error bars indicate SEM. Inset traces show sample fEPSPs recorded during the baseline period (a) and at 180 min after stimulation (b) at S1. Calibration: 1 mV, 10 ms.

with drug application to rmNP-supplemented KO hippocampal slices. Bath-applied KN93 with weak-HFS at S1 completely blocked the late associativity elicited by strong-HFS or strong-LFS at S0 in the stratum radiatum of rmNP-supplemented KO hippocampal slices (Fig. 6*B* and *F*). This result strongly suggested that neuropsin-dependent late associativity signalling occurred through CaMKII activity (KN93, 180 min after

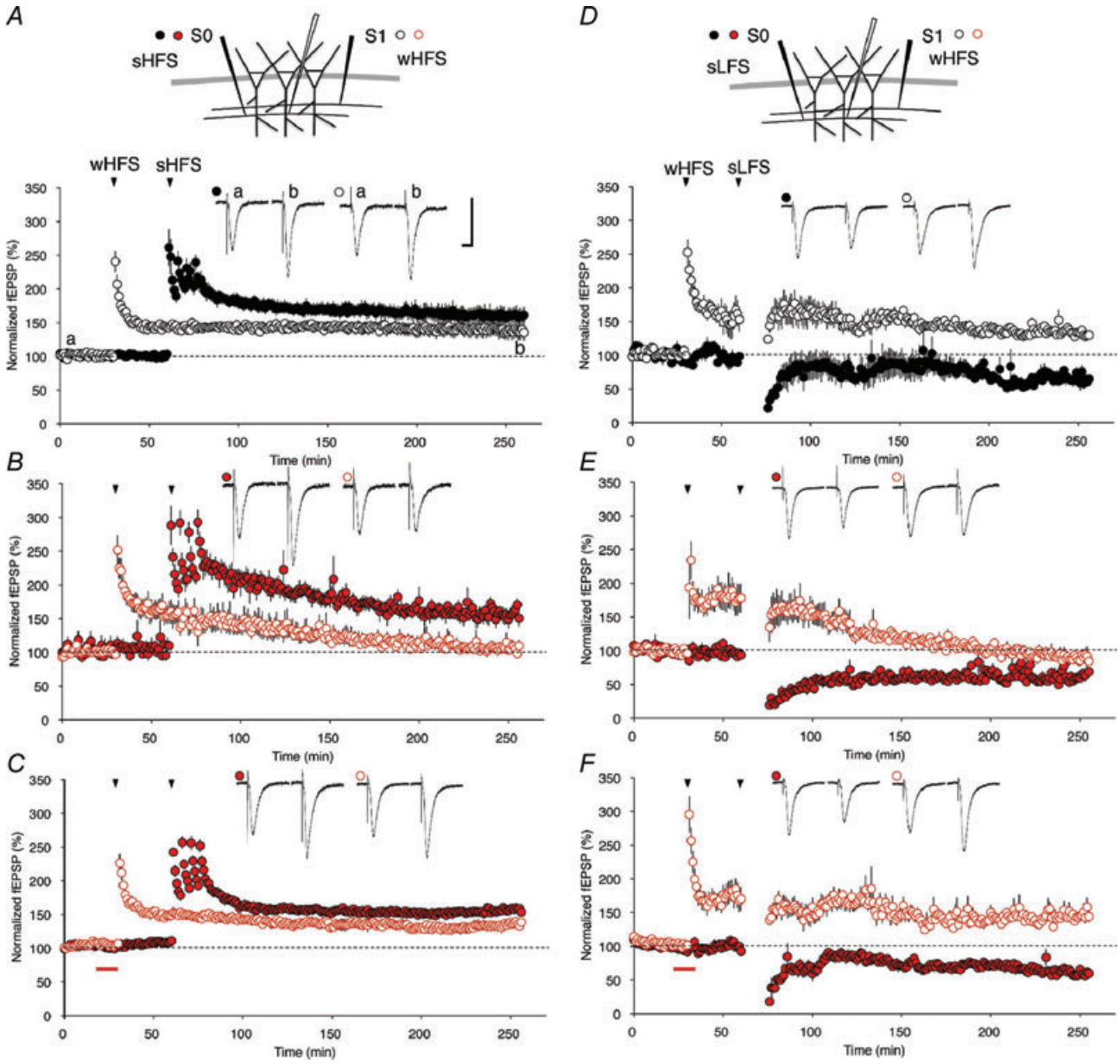


Figure 5. Performance of the weak before strong protocol: neuropsin is required for synaptic tagging and cross-tagging

A, schematic diagram of a hippocampal slice showing the positions of two stimulating electrodes (S0 and S1) and a single recording electrode placed in the stratum radiatum of area CA1. Weak-HFS at S1 (open circles) before strong-HFS at S0 (filled circles) resulted in stable L-LTP at S1 in WT hippocampal slices. *B,* by contrast, in KO hippocampal slices, fEPSP values were impaired after performing weak-HFS at S1 (red open circles) before strong-HFS at S0 (red filled circles). *C,* rmNP administration to KO slices reversed the induction of L-LTP at weakly stimulated S1. *D,* weak-HFS at S1 before strong-LFS at S0 resulted in stable L-LTP at S1 in WT hippocampal slices. *E,* by contrast, in KO hippocampal slices, fEPSP values were impaired by performing weak-HFS at S1 (red open circles) before strong-LFS at S0. *F,* rmNP administration to KO hippocampal slices reversed the induction of L-LTP at weakly stimulated S1. Red bar indicates rmNP administration. sHFS and wHFS indicate strong-HFS and weak-HFS, respectively. sLFS indicates strong-LFS. Error bars indicate SEM. Inset traces show sample fEPSPs recorded during the baseline period (a) and at 180 min after stimulation (b) at S1. Calibration: 1 mV, 10 ms.

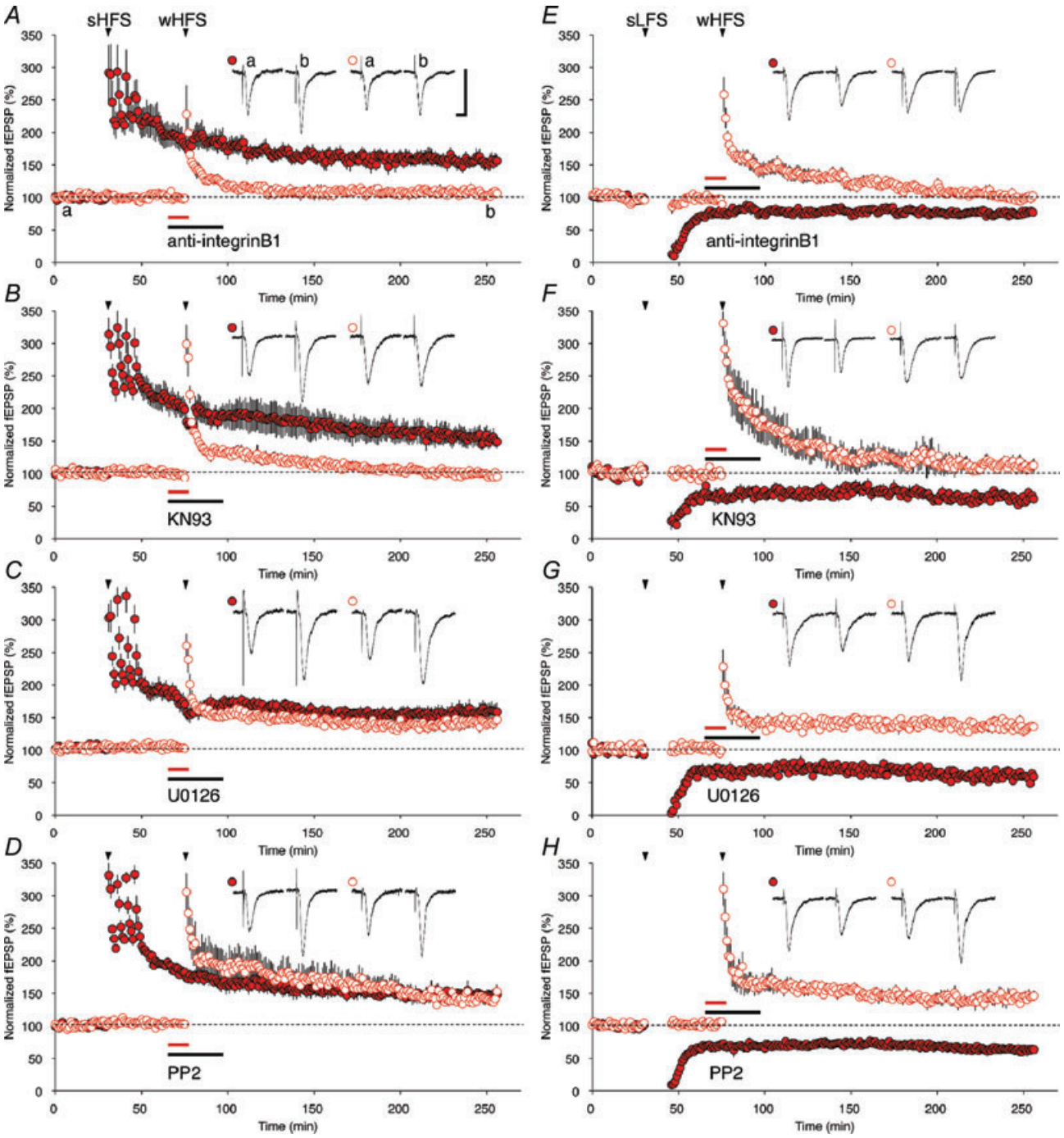


Figure 6. The role of integrin β1 and CaMKII in neuropsin-dependent late associativity
L-LTP or L-LTD transforms synaptic-tagged E-LTP synapses into persistent LTP in rmNP-treated hippocampal slices from KO mice (Fig. 1*D* and *H*). This neuropsin-dependent persistency involved integrin β1 and CaMKII signalling. *A*, similar to the experiment shown in Fig. 1*D*, L-LTP was induced at S0 (filled circles) by strong-HFS followed by the co-application of rmNP and integrin β1 neutralizing antibodies with weak-HFS at S1 (open circles). *B, C* and *D,* experiments similar to *A*, with the exception that kinase inhibitors KN93 (*B*), U0126 (*C*) and PP2 (*D*) were applied. *E,* protocol similar to that in *A*, except that strong-LFS was applied at S0. *F, G* and *H,* experiments similar to *E*, with the exception that kinase inhibitors KN93 (*F*), U0126 (*G*), and PP2 (*H*) were applied. Black horizontal bar represents the time period in which the drug was applied. Red short bar indicates rmNP administration. sHFS and wHFS indicate strong-HFS and weak-HFS, respectively. sLFS indicates strong-LFS. Error bars indicate SEM. Inset traces show sample fEPSPs recorded during the baseline period (a) and at 180 min after stimulation (b) at S1. Calibration: 1 mV, 10 ms.

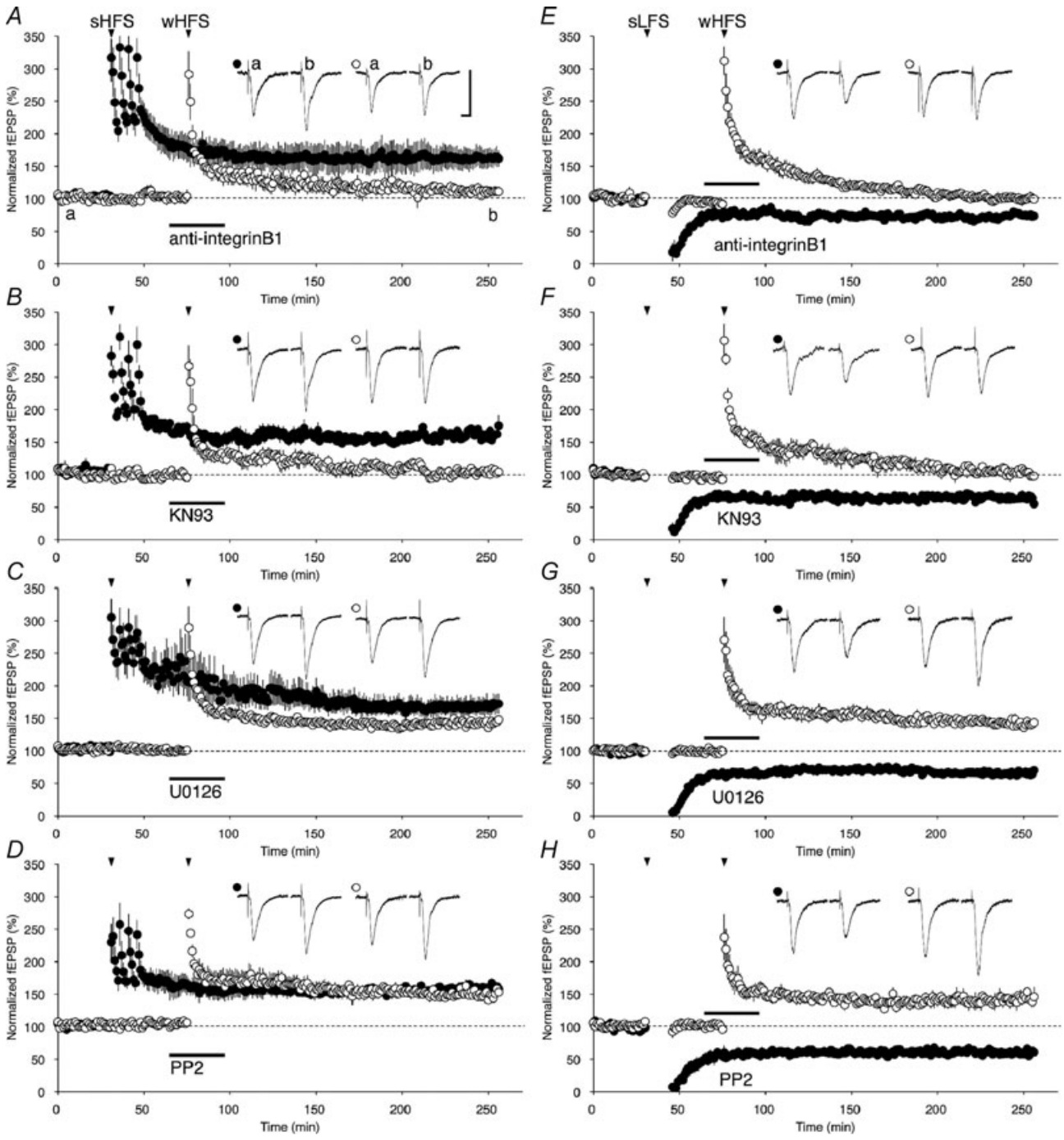


Figure 7. Role of integrin β1, CaMKII, ERK and Src in LTP-related synaptic late associativity
A, L-LTP induced at S0 by strong-HFS followed by the application of integrin β1 neutralizing antibodies with weak-HFS at S1 in WT hippocampal slices. *B, C* and *D,* experiments similar to *A*, with the exception that kinase inhibitors KN93 (*B*), U0126 (*C*), and PP2 (*D*) were applied. *E*, protocol similar to that in *A*, except that strong-LFS was applied at S0. *F, G* and *H,* experiments similar to *E*, with the exception that kinase inhibitors KN93 (*F*), U0126 (*G*) and PP2 (*H*) were applied. Horizontal bar represents the time period in which the drug was applied. sHFS and wHFS indicate strong-HFS and weak-HFS, respectively. sLFS indicates strong-LFS. Error bars indicate SEM. Inset traces show sample fEPSPs recorded during the baseline period (a) and at 180 min after stimulation (b) at S1. Calibration: 1 mV, 10 ms.

tetanus: synaptic tagging, 98 ± 5 at S1, $n = 5$ Fig. 6*B*; cross-tagging, 111 ± 8 at S1, $n = 6$ Fig. 6*F*). By contrast, ERK and Src-family tyrosine kinase inhibitors did not have any effect on neuropsin-dependent synaptic tagging or cross-tagging (180 min after tetanus: U0126 synaptic tagging, 147 ± 9 at S1, $n = 10$, Fig. 6*C*; U0126 cross-tagging, 135 ± 6 at S1, $n = 5$, Fig. 6*G*; PP2 synaptic tagging, 142 ± 6 at S1, $n = 5$, Fig. 6*D*; PP2 cross-tagging, 144 ± 5 at S1, $n = 5$, Fig. 6*H*). Integrin antibody and KN93 were confirmed to inhibit late associativity in WT hippocampal slices (Fig. 7).

However, it is still possible that neuropsin-dependent late associativity is due to the diffusion of extracellular neuropsin or other tag-setting related molecules, since neuropsin functions as an extracellular serine protease and either neuropsin itself, its cleavage products, or other diffusible molecules might readily diffuse from the strong tetanus-conditioned synaptic inputs to other synapses. To address this possibility, weak-HFS was performed after strong-HFS to investigate changes in late associativity following drug application to rmNP-supplemented KO hippocampal slices and WT hippocampal slices. Bath-applied KN93 together with a strong-HFS at S0 blocked L-LTP, but transformed E-LTP into a persistent L-LTP after weak-HFS at S1 in the stratum radiatum of rmNP-supplemented KO hippocampal slices and WT slices (synaptic tagging; 180 min after tetanus: WT, 144 ± 10 at S1, $n = 7$, Fig. 8*A*; KO + rmNP, 133 ± 4 at S1, $n = 4$, Fig. 8*B*). These experiments suggest that CaMKII, which is sensitive to this concentration of KN93, might play a major role in the LTP tag-setting process, whereas its role in the regulation of PRP might be more limited. KN93 not only blocked CaMKII, but also CaMKI and IV. We found that bath-applied higher doses of KN93 (10–20 μM) together with a strong-HFS at S0 blocked both L-LTP after strong-HFS at S0 and persistent L-LTP after weak-HFS at S1 in the stratum radiatum of rmNP-supplemented KO hippocampal slices (data not shown). These results are consistent with a previous report (Redondo *et al.* 2010). Therefore, we thought that low doses (1–5 μM) of KN93 would block the specific role of CaMKII in the tag-setting process and that high doses (10–20 μM) would have less selectivity and block both the tag-setting process and CaMKI and IV for the gene transcription of PRPs.

Overall, the present study demonstrated that neuropsin-dependent late associativity occurred in E-LTP-induced synaptic inputs through integrin and CaMKII signalling in apical dendrites.

Discussion

Neuropsin-dependent synaptic tagging

In this study, we demonstrated that neuropsin-dependent tagging occurs at synapses to which weak-HFS is delivered and can convert into persistent LTP when associated with either L-LTP or L-LTD, and that integrin and CaMKII signalling are involved in this tag-forming process. Although over a decade has passed since Frey and Morris introduced the synaptic tagging theory in 1997, the involvement of tags and their signalling mechanisms in late association has not yet been clarified (Frey & Morris, 1997; Morris, 2006; Barco *et al.* 2008; Frey & Frey, 2008). Two important processes are hypothesized to comprise synaptic tagging: (1) setting of the synaptic tag molecule triggered by a specific pattern of stimulation, and (2) a synaptic capture process in which a particular synaptic input is marked by a tag molecule, and newly synthesized PRPs are sorted into tagged synapses. Recently, there have been reports that the LTP-specific synaptic tag-setting process is mediated by CaMKII, whereas the LTD tag-setting process is mediated by ERK in apical dendrites of hippocampal CA1. Moreover, it has also been reported that protein kinase Mζ (PKMz), as LTP-specific PRP, is necessary and sufficient for maintaining LTP and that it mediates capture processes. Here, we report that neuropsin is a candidate molecule for the E-LTP tag-setting process involved in the initiation of synaptic tagging and cross-tagging. Together with previous reports, our findings suggest that the neuropsin-dependent E-LTP tag might use PKMz as a PRP for its maintenance, although additional studies will be needed to further elucidate this issue.

Behavioural aspects of neuropsin-dependent tagging in apical and basal dendrites

The neuropsin-dependent system is thought to be involved in the memory of low-stress behavioural tasks, particularly working memory, because its deficiency resulted in more marked physiological defects in low-stress memory tasks such as Y-maze and Morris water-maze tasks than in more stressful tasks (Tamura *et al.* 2006; Horii *et al.* 2008). We clearly induced neuropsin-dependent synaptic tagging in both intra-apical and intra-basal compartments, but did not observe cross-compartmentalization of two independent synaptic inputs between apical and basal dendrites. Moderate-HFS was able to induce cross-compartmentalization in both WT and neuropsin-KO hippocampal slices (data not shown), as previously described by Alarcon *et al.* (2006). These results suggest that neuropsin-dependent synaptic tagging is a local event, and that cross-compartmentalization as a cell-wide event is neuropsin independent.

Whether LTP at apical and basal dendrites differentially participates in spatial learning remains controversial because some investigators have reported that there are differences in the physiological and morphological properties between apical and basal dendrites of CA1

pyramidal neurons (Arai *et al.* 1994; Haley *et al.* 1996; Son *et al.* 1996; Cavus & Teyler, 1998; Leung & Shen, 1999; Kloosterman *et al.* 2001; Kramar & Lynch, 2003). Leuner *et al.* (2003) revealed that an increase in dendritic spine density by eyeblink conditioning-based association learning was specific to basal dendrites of CA1 pyramidal cells, but was not found in apical dendrites. Leung & Shen (1995, 1999) also suggested that NMDA receptor-dependent *in vivo* LTP for spatial learning contributes preferentially to basal CA1 dendrites *in vivo*. Although it is not yet clear which dendrite functions predominantly in neuropsin-dependent synaptic integration for spatial memory, apical and/or basal dendritic associativities may contribute to association memory through NMDA receptor signalling (Matsumoto-Miyai *et al.* 2003; Tamura *et al.* 2006).

Integrin β1 and CaMKII signalling are required for neuropsin-dependent tagging.

The use of neuropsin-KO hippocampal slices supplemented with rmNP in the present study provides strong evidence that neuropsin is involved in late associativity via integrin β1 and CaMKII. Integrin and CaMKII signals may have acted concurrently with neuropsin during the very narrow time window of rmNP application to KO hippocampal slices. Taken together with our previous results, these findings suggest that (a) a neuropsin-dependent processing event for extracellular/membrane proteins or the interaction of receptors and fragmented ligands cleaved by neuropsin produces a neuropsin-dependent tag in E-LTP-induced synapses, and (b) neuropsin–integrin signalling is followed by CaMKII signalling, or is working concomitantly with CaMKII signalling, and both types of signalling are crucial, but not ERK- or Src-kinase signalling, for the creation of the capturing entity of PRPs. Involvement of CaMKII in LTP-specific tag production or setting was also demonstrated by previous studies (Sajikumar *et al.* 2007; Redondo *et al.* 2010).

Cross-talk in integrin signalling for synaptic tag setting and persistent plasticity

Extracellular serine proteases and matrix metalloproteases (MMPs) have been studied as a hypothetical control system of neural plasticity, as well as learning and memory (Shiosaka & Yoshida, 2000; Kaczmarek *et al.* 2002; Molinari *et al.* 2003; Pang & Lu, 2004; Nagy *et al.* 2006; Tamura *et al.* 2006; Ethell & Ethell, 2007; Meighan *et al.* 2007; Ishikawa *et al.* 2008; Wang *et al.* 2008). In these studies, the major function of the proteases was considered to be the processing (or shedding) of extracellular matrix/cell adhesion molecules and membrane-associated receptors. For instance, tissue plasminogen activator (tPA) and MMPs directed against processing targets other than the neuropsin-dependent system, which is related to fast and early plasticity, may be involved in later plasticity function. Physiological and behavioural studies using genetically manipulated mice or specific inhibitors of protease activity demonstrated that tPA- and MMP-mediated modification is predominant in persistent plasticity, particularly in the structural modification of spine morphology. MMP-9 drives dendritic spine enlargement via integrin β1 signalling and functions to sustain L-LTP (Wang *et al.* 2008; Michaluk *et al.* 2009).

As shown in the present study, neuropsin-dependent associativity also functions via integrin β1; thus, intriguingly, neuropsin signalling and MMP-9 signalling

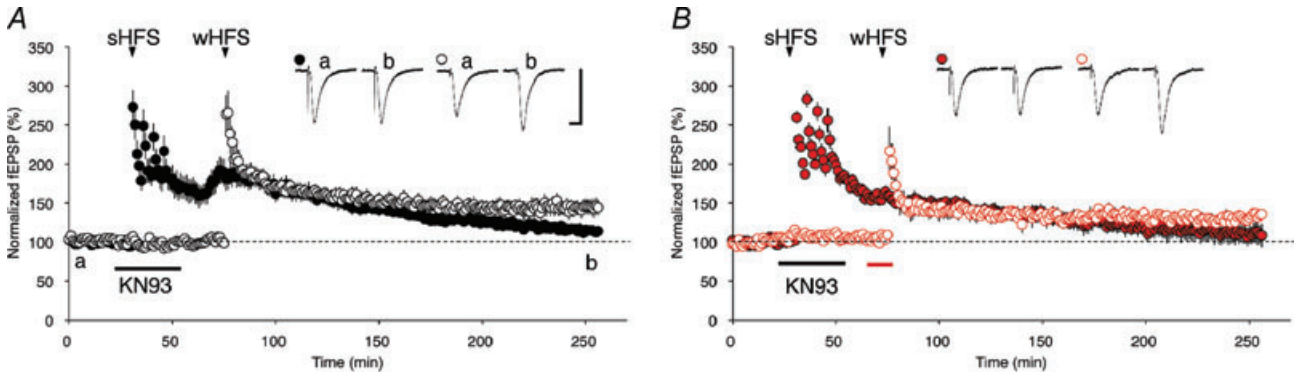


Figure 8. The specific role of CaMKII in the tag-setting process
Strong-HFS at S0 in the presence of KN93 induces LTP that decays to the baseline over 180 min, whereas an independent set of weak-HFS synaptic inputs results in stable potentiation for 180 min after tetanus at S1 in WT (*A*) and KO + rmNP (*B*) hippocampal slices. Black horizontal bar represents the time period in which the KN93 was applied. Red short bar indicates rmNP administration. sHFS and wHFS indicate strong-HFS and weak-HFS, respectively. Error bars indicate SEM. Inset traces show sample fEPSPs recorded during the baseline period (a) and at 180 min after stimulation (b) at S1. Calibration: 1 mV, 10 ms.

may converge into the same integrin system in spite of having dissimilar functions. Neuropsin is related to E-LTP and exploratory behaviour (Tamura *et al.* 2006; Horii *et al.* 2008), while MMP-9 is related to L-LTP and escape learning (Nagy *et al.* 2006, 2007). However, it is not yet clear how and why such dissimilar functions are transmitted through the same integrin signal, which promotes tag setting and persistency in LTP during late associativity. Future studies investigating neuropsin and other synaptic plasticity-related proteases will continue to expand our understanding of the regulation of late associativity as complex information-processing systems.

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Author contributions

The experiments were performed at the Laboratory of Functional Neuroscience, Nara Institute of Science and Technology, Japan. Y.I. and S.S. conceived and designed the experiments. Y.I. collected, analysed and interpreted the data. Y.I., H.T. and S.S. drafted the article or revised it critically for important intellectual content. All authors approved the final version of the manuscript.

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