

# Role of neuropsin in formation and maturation of Schaffer-collateral L1cam-immunoreactive synaptic boutons

Yukiko Nakamura, Hideki Tamura, Kazuhiro Horinouchi and Sadao Shiosaka\*

Division of Structural Cell Biology, Nara Institute of Science and Technology, 8916-5, Takayama, Ikoma, Nara 630-0192, Japan

\*Author for correspondence (e-mail: sshiosak@bs.naist.jp)

Accepted 19 December 2005

Journal of Cell Science 119, 1341-1349 Published by The Company of Biologists 2006

doi:10.1242/jcs.02862

## Summary

We report that neuropsin is involved in the synaptogenesis/maturation of orphan and small synaptic boutons in the Schaffer-collateral pathway. Most non-synaptic orphan boutons and a number of immature small synaptic boutons expressed the cell adhesion molecule L1 in presynaptic Schaffer-collateral terminals, whereas mature large boutons on mushroom spines were devoid of L1. The number of L1-immunoreactive boutons was markedly higher in neuropsin-deficient mice than in wild-type mice, whereas there were far fewer mature large boutons. L1-immunoreactive boutons were hypertrophied in the mutant mice. When a recombinant active neuropsin was microinjected into the mutant hippocampus, the

number of immunoreactive synaptic boutons reverted to wild-type levels after one day. These results strongly suggest that enzymatically active neuropsin allows a maturational change of L1-immunoreactive small boutons, both orphan and synaptic, and this step may be important in synaptic plasticity based on activity-dependent structural change.

Supplementary material available online at  
<http://jcs.biologists.org/cgi/content/full/119/7/1341/DC1>

Key words: KLK8, Serine protease, Cell adhesion molecule, Synapse, Orphan bouton, Synaptogenesis

## Introduction

The dynamic regulation of synaptic strength by neural activity, i.e. rapid quantitative and qualitative changes in synapses, is fundamental to aspects of neural plasticity such as learning and memory. A number of studies have concentrated on the glutamatergic receptor function and signal transduction (Collingridge and Bliss, 1995). It has recently been suggested that an activity-dependent modification of extracellular molecules that occurs in the vicinity of asymmetrical synapses modulates synaptic morphology (Benson et al., 2000; Shiosaka and Yoshida, 2000). Spines forming asymmetric synapses are morphologically divided into at least two types – thin and mushroom; the former have a much smaller head than the latter (Fiala and Harris, 1999). Matsuzaki et al. (Matsuzaki et al., 2004) have shown, using two-photon photolysis of caged glutamate at single spines, that glutamate release induces a rapid and selective enlargement of the stimulated spine. Spine head size is clearly related to the induction of long-term potentiation (LTP), which is transient in large mushroom spines but persistent in small spines. The enlargement of spines is associated with the accumulation of AMPA ( $\alpha$  amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors, implying potentiation of synaptic efficacy. Takumi et al. (Takumi et al., 1999) revealed by immunoelectron microscopy that the postsynaptic density (PSD) of small synapses lacks AMPA receptors, and thus are expected to be ‘silent synapses’. It is also possible that small spines are preferential sites for the induction of LTP, whereas larger spines represent physical traces of long-term memory (Matsuzaki et al., 2004).

Collectively, small synapses in contact with small spines with small PSDs are considered to be plastic structures ready for the acquisition and consolidation of memory. However, the molecules that participate in these synaptic structural changes and the mechanisms involved in synaptogenesis/maturation have not yet been identified.

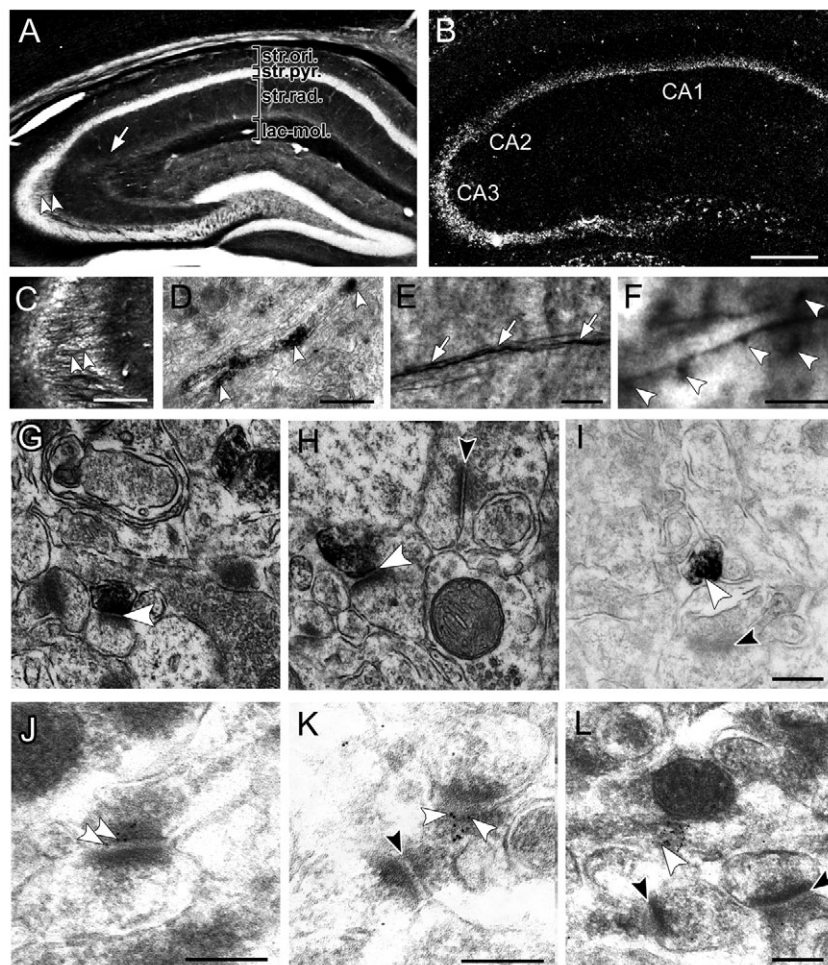
The secretory protease neuropsin is associated with activity-dependent neural plasticity, i.e. LTP and kindling epileptogenesis (Okabe et al., 1996; Momota et al., 1998; Komai et al., 2000). When recombinant active neuropsin (actNP) protein is bath-applied to hippocampal slices, LTP is facilitated, whereas application of an anti-neuropsin antibody that neutralizes the protease activity results in a deterioration of LTP (Momota et al., 1998; Komai et al., 2000). In the neuropsin-deficient mice, the number of non-synaptic orphan boutons is significantly increased, whereas asymmetrical mature type synapses are decreased (Hirata et al., 2001). It was recently shown that neural excitation triggers a transient activation of proneuropsin (zymogen) to cleave full-length cell adhesion molecule L1 (L1<sub>200</sub>) into L1<sub>180</sub> (180-kDa fragment of L1) in vitro (Matsumoto-Miyai et al., 2003). L1 has also been implicated in the generation of LTP, because application of L1 antibody or recombinant L1 fragment to hippocampal slices results in the decay of LTP (Luthi et al., 1994). These results show that neuropsin might participate in synaptic plasticity, including the regulation of synaptic transmission and synaptic structure, via cleavage of L1. However, the mechanism by which neuropsin and L1 influence synaptic plasticity is not known. In the present study, we revealed that immature orphan

and presynaptic small boutons containing L1 undergo a neuropsin-dependent morphological change, becoming L1-negative asymmetrical boutons on the large mushroom-type spines. Neuropsin and L1 may therefore associate to regulate synaptogenesis/maturation of synaptic structures.

## Results

L1 is localized to the presynaptic terminal boutons of the Schaffer-collateral pathway. Immunoreactive L1 (L1ir) was observed in axons which could be traced to CA3 pyramidal neurons (Fig. 1A, arrow, arrowheads) (Matsumoto-Miyai et al., 2003). In situ hybridization analysis demonstrated strong signals for L1 mRNA in the CA1-CA3 pyramidal neurons, implying that these cells produce L1 (Fig. 1B). L1ir was found in the axons of CA3 pyramidal neurons (Fig. 1C,D), axon bundles along the Schaffer-collateral pathway (Fig. 1E). In the Schaffer-collateral terminal area of the CA1 subfield, L1ir was detected in the axonal processes and in numerous protrusions from the axons (Fig. 1F, arrowheads). Electron microscopic (EM) observation of immunoreacted tissue sections using two different markers – diaminobenzidine and colloidal gold – revealed that L1ir was localized in the cytoplasm of small synapses (Fig. 1G,H,J,K) and orphan boutons (Fig. 1I,L), and associated with the presynaptic membrane of asymmetrical synapses that had thick PSDs (Fig. 1G,H,J,K, open arrowhead). When colloidal gold was used as a marker, clustering of gold particles on the presynaptic membrane was clearly visible on the presynaptic densities (Fig. 1J,K, open arrowheads). Thus, we conclude that L1 protein is produced in the pyramidal cell body, transported by anterograde axonal transport through the axon, and accumulates in the presynaptic structure of terminal boutons.

All the positive presynaptic boutons had small heads containing a few synaptic vesicles, and were in contact with thin spines having smaller PSDs than L1ir-negative asymmetrical synapses (see Fig. 3C). Three-dimensional (3D) reconstruction of stacks of EM images acquired from consecutive ultrathin sections enabled us to see L1ir orphan and small synaptic boutons (Fig. 2). L1ir was distributed beneath the presynaptic membrane (Fig. 2Ab,Bb, red). Typical L1ir-negative axonal boutons had a large head contacting with mushroom-type large spines (Fig. 2Ac,Bc). Measurement of the spine head area and PSD length of all synapses present in a fixed square of a single-plane EM image showed that L1ir synapses were much smaller than L1ir-negative synapses (Fig. 3A-C, L1 versus



**Fig. 1.** L1 localization to presynaptic terminals in the Schaffer-collateral pathway. (A) Overview of staining by anti-L1 antibody with DAB in the hippocampus of adult mice. The arrowheads indicate strongly stained black bundles from CA3 soma and immunoreactive fibers projected toward the stratum radiatum in the CA1 subfield (arrow). Stratum oriens (str. ori.), stratum pyramidale (str. pyr.), stratum radiatum (str. rad.) and lacunosum-moleculare (lac-mol.) are indicated. (B) Expression of L1 mRNA in hippocampus detected by in situ hybridization. Intense hybridization signals in the pyramidal layer, especially in CA3 pyramidal cells, appear as silver grains under dark-field illumination. (C) Higher magnification of L1ir axon bundles from CA3 soma marked by arrowheads in A. (D) Immunoelectron microscopic analysis of regions marked by arrowheads in A and C. L1ir is observed on and in axons (arrowheads). (E) Higher magnification of L1ir fibers marked by arrow in A. Some fasciculated axons show L1ir (arrows). (F) Higher magnification of an L1ir axon in the CA1 stratum radiatum. Arrowheads indicate L1ir boutons on axons. (G-I) Immunoelectron microscopic localization of L1 by pre-embedding staining in the CA1 stratum radiatum. L1ir is distributed in presynaptic sites of asymmetrical synapses (open arrowheads). L1ir was observed in orphan boutons containing a few vesicles (I). Closed arrowheads are L1ir-negative asymmetrical synapses. (J-L) Immunogold labeling of L1ir in CA1 stratum radiatum. Gold particles (10 nm, indicated by open arrowheads) are associated with the presynaptic membrane, the presynapse interior (J,K), and orphan boutons (L). Closed arrowheads indicate L1ir-negative asymmetrical synapses. Bars, 300  $\mu$ m (A,B); 100  $\mu$ m (C); 1  $\mu$ m (D); 5  $\mu$ m (E,F); 300 nm (I for G-I; J-L).

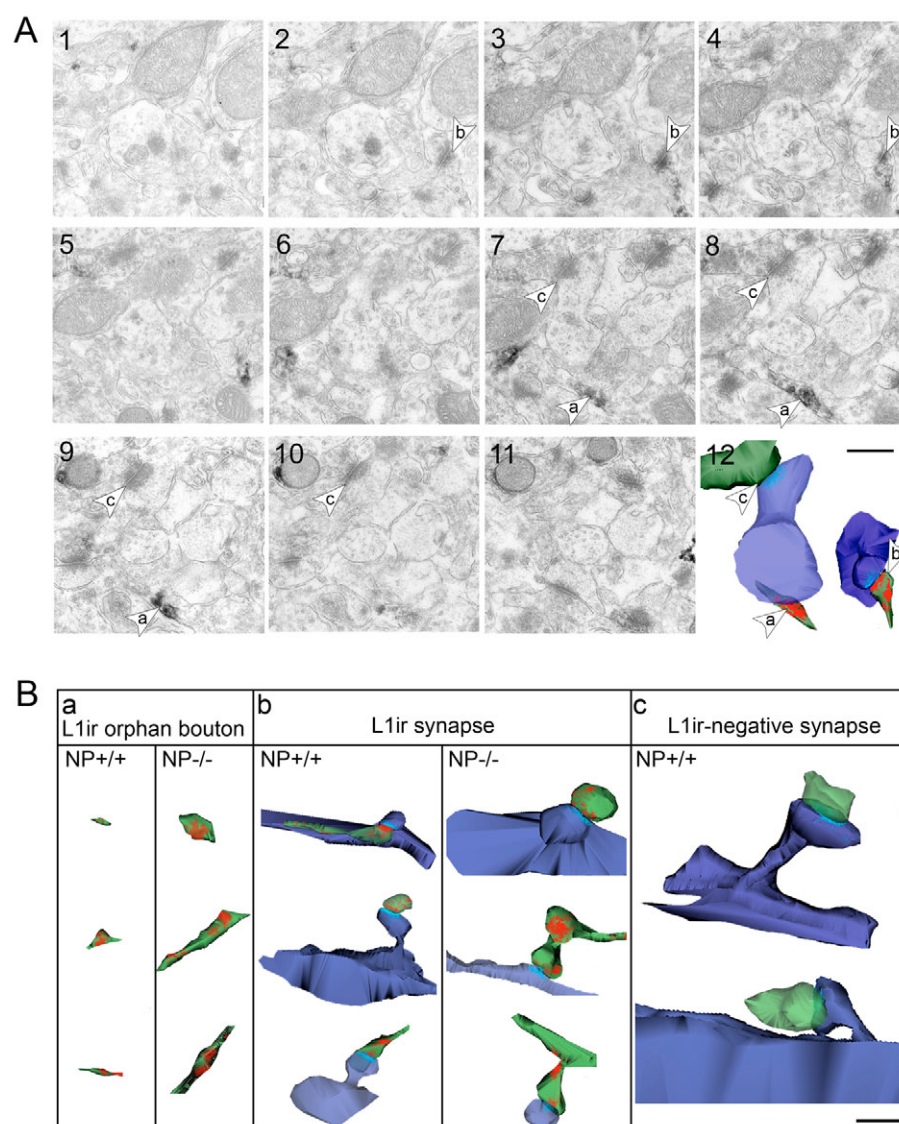
L1neg). From their morphology, L1ir orphan or synaptic boutons were considered to be functionally immature or temporary structures for synaptogenesis/maturation of synapses (see Discussion).



### Increased number of L1ir orphan and synaptic boutons in NP<sup>-/-</sup> mice

Since the activation of proneurotrophin zymogen and subsequent cleavage of L1 occur in the hippocampus in an activity-dependent manner (Matsumoto-Miyai et al., 2003), it was hypothesized that neurotrophin converts immature (L1ir) into mature (L1ir-negative) synapses. Therefore, we explored whether neurotrophin-deficient (neurotrophin<sup>-/-</sup>) mice exhibit quantitative abnormalities in the immature (L1ir) and mature (L1ir-negative) boutons. Low-magnification electron micrographs clearly revealed larger numbers of L1ir orphan and synaptic boutons in the stratum radiatum of the CA1 subfield in the NP<sup>-/-</sup> mice than in the NP<sup>+/+</sup> mice (Fig. 4A). L1ir deposits in the orphan and synaptic boutons were markedly denser in NP<sup>-/-</sup> than in NP<sup>+/+</sup> mice (Fig. 4A). Western blot analysis showed that the marked increase in band density of L1ir in NP<sup>-/-</sup> than NP<sup>+/+</sup> mouse hippocampus (Fig. 4B). These findings strongly suggested that L1ir accumulates in both structures in the NP<sup>-/-</sup> mice. To examine the difference in L1ir synaptic localization between NP<sup>-/-</sup> and NP<sup>+/+</sup> mice, L1ir synaptic boutons were plotted on a montage of 25 low-magnification ( $\times 6000$ ) electron micrographs covering all layers of the stratum radiatum and lacunosum moleculare. The density and topographic localization clearly differed between the two (Fig. 5A). L1ir synaptic boutons were more abundant, particularly in the proximal one-third of the stratum radiatum, in the NP<sup>-/-</sup> than in the NP<sup>+/+</sup> mice (Fig. 5A). L1ir orphan boutons were much more numerous in the NP<sup>-/-</sup> mice, and deposits of immunoreactivity were much denser in these than in the NP<sup>+/+</sup> mice (Fig. 4A, asterisks).

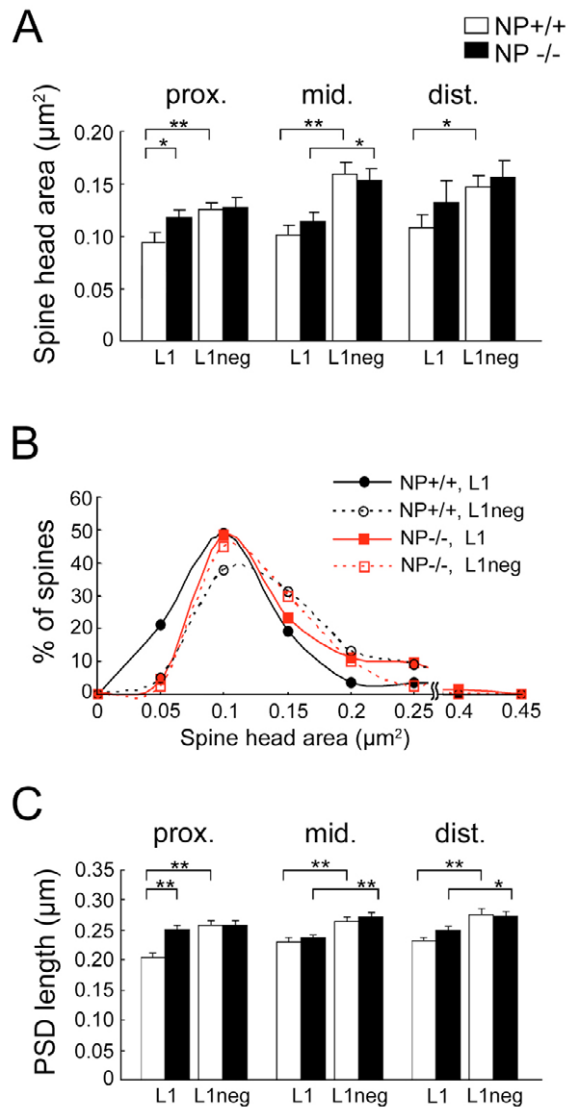
To confirm the results of visual inspection, we next analyzed NP<sup>-/-</sup> and NP<sup>+/+</sup> hippocampus using quantitative immunoelectron microscopy (Fig. 5B,C). L1ir small and L1ir-negative large asymmetrical synapses were identified based on synaptic characteristics (i.e. the presence of synaptic vesicles, PSDs, an apposed synaptic membrane and a uniform synaptic cleft). The number and percentage of L1ir small synapses were significantly higher in the NP<sup>-/-</sup> compared with the NP<sup>+/+</sup> mice (Fig. 5B;  $9.27 \pm 1.75\%$  versus  $4.87 \pm 0.65\%$ ,  $P < 0.05$ ). The difference was marked in the proximal third of the stratum radiatum ( $8.08 \pm 1.19\%$  versus  $3.82 \pm 0.85\%$ ,  $P < 0.01$ ) (Fig. 5B; Table 1). The percentage of L1ir/total synapses was approximately twofold higher in the mutant. On the other hand, the total number of asymmetrical synapses (L1ir and L1ir-



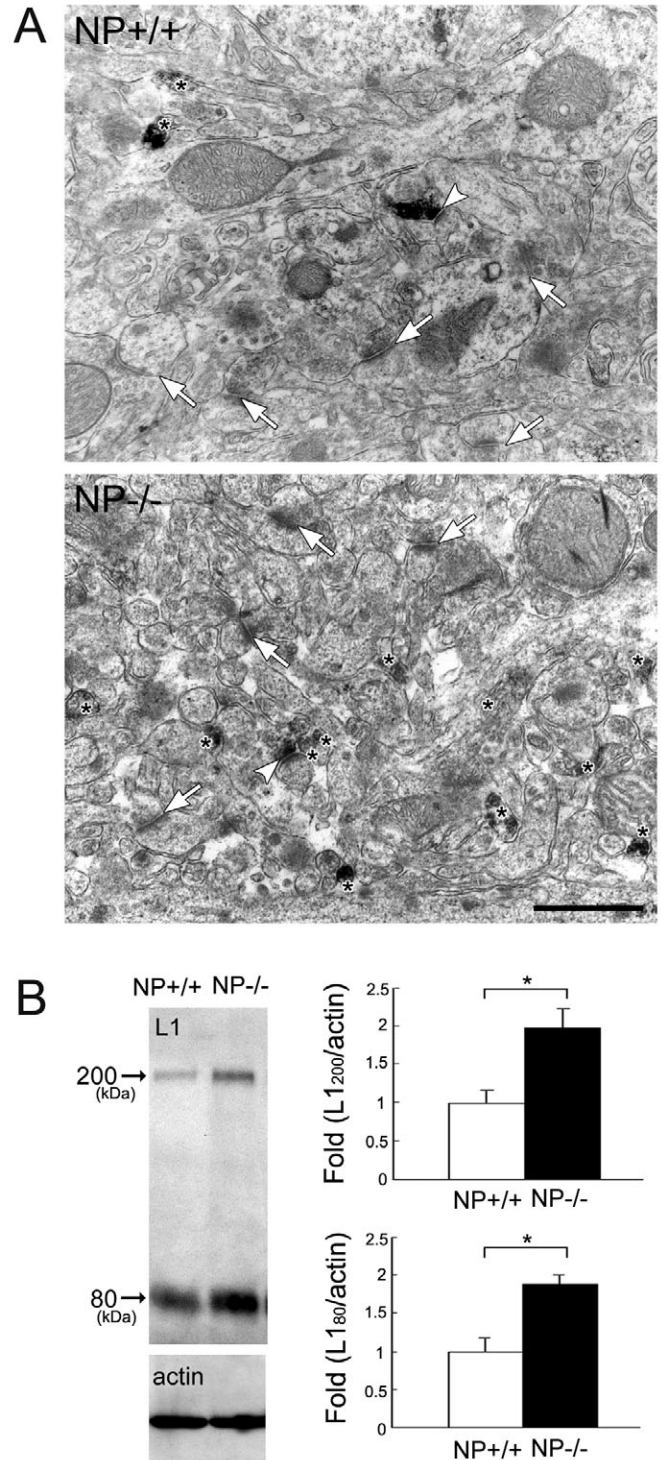
**Fig. 2.** Morphology of L1ir and L1ir-negative synapses. (A) Electron micrographs of consecutive ultrathin sections (1–11) and three-dimensional reconstruction demonstrating the L1ir orphan bouton (a), L1ir synapse (b) and L1ir-negative synapse (c) of the same microscopic field in NP<sup>+/+</sup> mice (12). (B) Complete three-dimensional reconstruction of L1ir orphan boutons (a), L1ir synapses (b) and L1ir-negative synapses (c) in NP<sup>+/+</sup> and NP<sup>-/-</sup> mice. Reconstructed presynapses are green, postsynapses are blue, their PSDs are light blue and L1-immunoreactive sites are red. Bars, 600 nm for all panels.

negative) in the stratum radiatum was significantly lower in the proximal layer of the NP<sup>-/-</sup> hippocampus (Fig. 5C) (Hirata et al., 2001). Moreover, the majority of orphan boutons were L1ir and the number in the neurotrophin-deficient mice was also increased twofold (Table 1). Intriguingly, the increase in L1ir orphan boutons was most apparent in the proximal third of the stratum radiatum (Fig. 5D, Table 1).

We examined the input-output (I–O) relationship of Schaffer collateral-CA1 synapses (supplementary material Fig. S1). The I–O curves for fEPSP with 500 and 600  $\mu$ A were different between NP<sup>-/-</sup> and NP<sup>+/+</sup> mice. These data also suggest that the number of synapses is lower in NP<sup>-/-</sup> mice than NP<sup>+/+</sup> mice. These results, combined with our EM studies imply that the



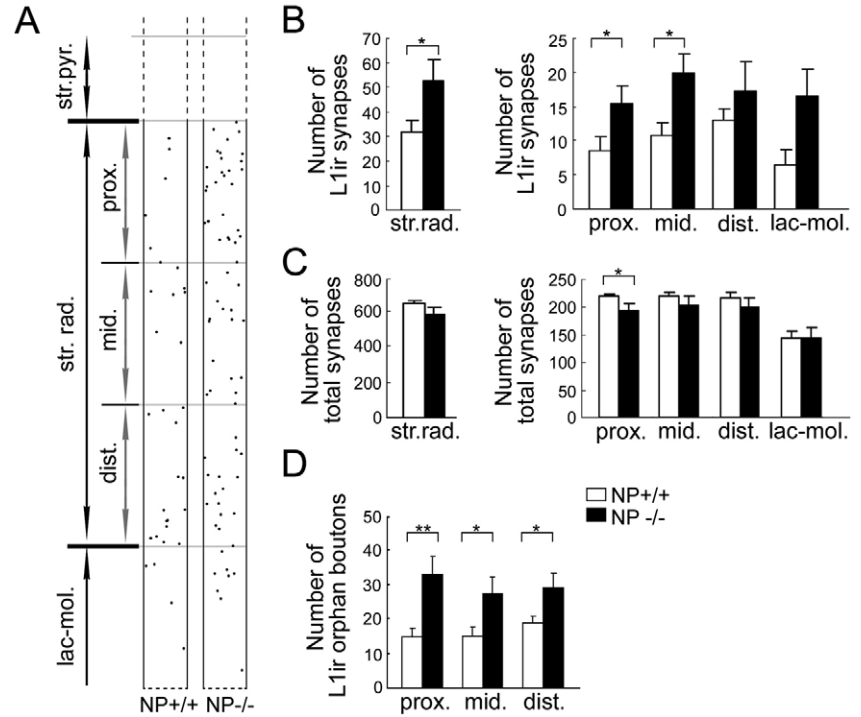
**Fig. 3.** Shift from smaller to larger L1ir synapses in NP<sup>-/-</sup> mice. (A) The stratum radiatum was divided into three regions, proximal (prox.), middle (mid.) and distal (dist.), according to distance from the CA1 soma. In NP<sup>+/+</sup> mice (open bars), the mean cross-sectional spine head area of L1ir (L1) synapses is significantly smaller than that of L1ir-negative (L1neg) synapses in all regions of the stratum radiatum. The mean cross-sectional spine head area of L1ir synapses is larger in NP<sup>-/-</sup> mice (filled bars) than in NP<sup>+/+</sup> mice (open bars), especially in the proximal region. \**P*<0.05; \*\**P*<0.001. (B) The distribution of spine head area in the proximal region of L1ir synapses and L1ir-negative synapses in NP<sup>+/+</sup> and NP<sup>-/-</sup> mice. In NP<sup>+/+</sup> mice, L1ir synapses were significantly smaller than L1ir-negative synapses (Kolmogorov-Smirnov test, *P*<0.03). In NP<sup>-/-</sup> mice, the distribution of L1ir synapses shifted to a larger size, comparable with that of L1ir-negative synapses. (C) The mean cross-sectional PSD length of L1ir synapses was significantly smaller than that of L1ir-negative synapses in NP<sup>+/+</sup> (open) and NP<sup>-/-</sup> (closed) mice, except in the proximal region where the length of L1ir synapses in NP<sup>-/-</sup> mice was significantly larger than that in NP<sup>+/+</sup> mice and comparable with the level in L1ir-negative synapses (*P*>0.55). \**P*<0.05; \*\**P*<0.001.



**Fig. 4.** Change in the distribution of L1ir sites in NP<sup>-/-</sup> mice. (A) Low-power immunoelectron micrographs of the proximal region of the CA1 stratum radiatum in NP<sup>+/+</sup> (upper panel) and NP<sup>-/-</sup> (lower panel) mice. There are more L1ir synaptic (arrowheads) and L1ir orphan boutons (asterisks) in NP<sup>-/-</sup> mice than in NP<sup>+/+</sup> mice. Arrows show L1ir-negative synapses. Bar, 1 μm. (B) Western blot analysis of L1 in hippocampus of NP<sup>+/+</sup> and NP<sup>-/-</sup> mice. Both the 80 and 200 kDa L1ir bands were detectable. Actin served as internal control for protein loading. The graph shows the quantification of band intensities of L1<sub>200</sub> and L1<sub>80</sub>. The band density of L1 was significant increase in NP<sup>-/-</sup> mice. \**P*<0.01.



**Fig. 5.** Increased number of L1ir synaptic and orphan boutons in NP<sup>-/-</sup> mice. (A) Typical distribution of L1ir synapses (dots) in NP<sup>+/+</sup> (left) and NP<sup>-/-</sup> (right) mice in the CA1 subfield. L1ir synaptic boutons are plotted on a montage of 25 low-power ( $\times 6000$ ) electron micrographs of stratum pyramidale (str.pyr.), stratum radiatum (str.rad.) and stratum lacunosum moleculare (lac-mol.). The stratum radiatum (str. rad.) was divided into three regions, proximal (prox.), middle (mid.) and distal (dist.), according to the distance from CA1 soma. (B) The number of asymmetrical L1ir synapses was counted in NP<sup>+/+</sup> (open bars) and NP<sup>-/-</sup> (closed bars) mice. The left graph shows the number of L1ir synapses in a fixed area ( $13.0 \times 219.6 \mu\text{m}$ ) of the CA1 stratum radiatum, and the right graph shows the number in each region according to the distance from CA1 soma in a fixed area ( $13.0 \times 73.2 \mu\text{m}$ ). The mean number of L1ir synapses was significantly higher in NP<sup>-/-</sup> mice.  $*P < 0.05$ . (C) Total number of asymmetrical synapses containing L1ir and L1ir-negative synapses was counted in NP<sup>+/+</sup> (open bars) and NP<sup>-/-</sup> (closed bars) mice. The left graph shows the number of synapses in the stratum radiatum in a fixed area ( $13.0 \times 219.6 \mu\text{m}$ ), and the right shows the number in each region depending on the distance from the CA1 soma in a fixed area ( $13.0 \times 73.2 \mu\text{m}$ ). The mean number of total synapses was significantly lower in the proximal region of NP<sup>-/-</sup> mice.  $*P < 0.05$ . (D) The mean number of L1ir orphan boutons was significantly higher in NP<sup>-/-</sup> (closed bars) mice than NP<sup>+/+</sup> (open bars) mice in the proximal region in a fixed area ( $13.0 \times 16.5 \mu\text{m}$ ).  $*P < 0.05$ ;  $**P < 0.01$ .



deficiency of neurotrophin induced an accumulation of L1ir orphan and small synaptic boutons, coincident with impairment of the formation of mature synapses.

**Hypertrophied orphan and small synaptic boutons were often found in the neurotrophin-deficient mouse**  
Since the quantitative measurements showed that L1ir orphan and synaptic boutons were markedly increased in the NP<sup>-/-</sup> mice, L1ir boutons were further assessed by measurement of

the size of spine head area and PSD length in comparison with wild-type mice (Fig. 2Ba,b, NP<sup>-/-</sup> versus NP<sup>+/+</sup>; Fig. 3A-C). In the NP<sup>-/-</sup> mice, hypertrophy of orphan and synaptic boutons was apparent in the proximal one-third of the stratum radiatum, but not in the other layers (Fig. 3A,C). These results suggest that remodeling of synaptic structure occurs most frequently in this area, causing the hypertrophy and increase of L1ir presynaptic boutons in the mutant mice. Collectively, quantitative and qualitative examination of the L1ir structures

**Table 1. Number and percentage of L1ir orphan and synaptic boutons in the stratum radiatum of NP<sup>-/-</sup> and NP<sup>+/+</sup> mice**

		Proximal		Middle		Distal		n
		Number	%	Number	%	Number	%	
Orphan boutons								
NP <sup>+/+</sup>	L1ir	70.5±11.5	(83.4±5.2)	62.6±10.6	(64.6±4.6)	83.8±10.8	(71.2±3.3)	8
	L1ir-neg	11.5±3.1	(16.6±5.2)	31.3±5.0	(35.4±4.6)	32.5±4.7	(28.8±3.3)	8
	Total	82.1±10.7		93.9±11.3		116.3±12.2		8
NP <sup>-/-</sup>	L1ir	142.6±28.8*	(83.7±3.4)	134.2±28.9*	(74.4±4.6)*	145.9±24.3*	(83.9±2.5)**	8
	L1ir-neg	19.2±2.0	(16.3±3.4)	28.9±3.8	(25.6±4.6)*	24.0±2.6	(16.1±2.5)**	8
	Total	161.8±28.8*		163.0±35.8*		169.9±24.9		8
Synaptic boutons								
NP <sup>+/+</sup>	L1ir	9.0±2.1	(3.8±0.9)	11.3±2.0	(4.9±0.8)	13.7±1.9	(6.1±0.9)	7
	L1ir-neg	223.1±3.8	(96.2±0.9)	219.7±8.4	(95.1±0.8)	214.6±11.4	(93.9±0.9)	7
	Total	232.1±4.3		231.1±8.8		228.3±11.6		7
NP <sup>-/-</sup>	L1ir	16.4±2.7*	(8.1±1.2)**	21.0±2.6*	(10.4±1.8)**	18.2±4.5	(9.2±2.7)	7
	L1ir-neg	188.4±12.2**	(91.9±1.2)**	191.9±17.7	(89.6±1.8)**	191.0±18.3	(90.8±2.7)	7
	Total	204.8±12.8*		212.9±17.0		209.1±17.5		7

The numbers of boutons per 1000  $\mu\text{m}^2$  in proximal, middle and distal regions from the CA1 soma were counted (mean  $\pm$  s.e.m.) and the percentages of L1ir and L1ir-negative (L1ir-neg) synaptic and orphan boutons per total (L1ir and L1ir-neg) are shown. Significant changes in the number and percentage of L1ir synaptic and orphan boutons between NP<sup>-/-</sup> and NP<sup>+/+</sup> mice (Student's unpaired *t*-test) are indicated.  $*P < 0.05$ ;  $**P < 0.01$ .

strongly support the idea that neuropsin is involved in the conversion of L1ir orphan and synaptic boutons into L1ir-negative large synaptic boutons, particularly in the proximal part of the stratum radiatum.

### Injection of active neuropsin rescued the quantitative but not qualitative abnormality in neuropsin-deficient hippocampus

The prominent accumulation and hypertrophy of L1ir orphan and synaptic boutons and the decrease in the number of L1ir-negative mature synapses in the mutant signify a conversion of L1ir orphan and synaptic boutons into mature synapses that lack L1ir. This issue was examined further by administering recombinant active neuropsin (actNP) into the NP<sup>-/-</sup> mouse hippocampus; if neuropsin-dependent synaptic conversion occurs as in adults, supplying exogenous actNP to adult mutant mice might rescue the abnormality. Since neuropsin is known to act extracellularly (Momota et al., 1998; Komai et al., 2000; Oka et al., 2002), recombinant actNP was microinjected into the stratum radiatum of the CA1 subfield in vivo. To assess the degree of spread of the actNP, an FITC-conjugated recombinant actNP was injected and found to spread over most of the stratum radiatum (Fig. 6A). After the potentiating effect of neuropsin on extracellular postsynaptic potential (EPSP) was measured in vivo, the same mice were processed for immunoEM: i.e. 1 day after EPSP measurement, followed by quantitative immunoEM analysis of the proximal third of the stratum radiatum. When recombinant actNP was administered by a single injection at a physiological dose, the number and percentage of L1ir synapses were significantly reduced to the levels seen in untreated wild-type mice (Fig. 6B), although the total synaptic number was similar to the group receiving ACSF injections ( $187.6 \pm 10.5$  for ACSF versus  $198.7 \pm 10.5$  for actNP). Thus, injected recombinant actNP mimics the function of endogenous actNP and can convert L1ir synapses into L1ir-negative mushroom spine synapses.

### Light microscopic observation of postsynaptic neurons in NP<sup>-/-</sup> mice

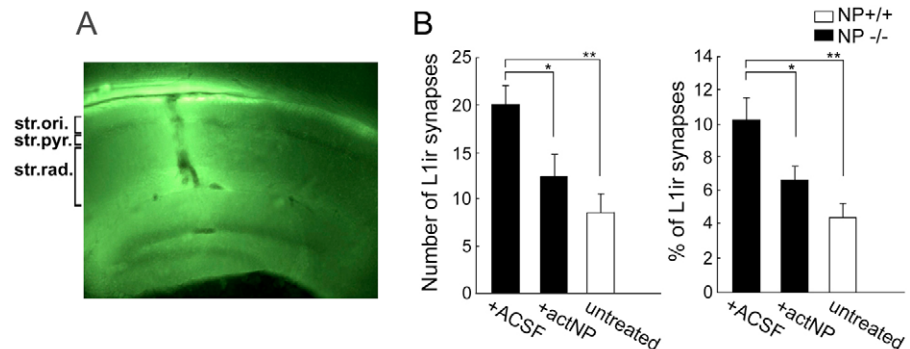
The finding that synaptic number and morphology was abnormal in NP<sup>-/-</sup> mice suggested that there might be changes in the postsynaptic neurons in the mutants. Therefore, we examined the density of spines by microinjecting Lucifer yellow, a fluorescent dye, into pyramidal neurons of the CA1

subfield and by 3D observation using a two-photon microscope. However, there were no apparent differences in the number of spines in the dendrites between the two genotypes (Fig. 7A-C), nor in the morphology of dendritic arbors or the length of the apical dendrites (Fig. 7A). These observations were confirmed using the Golgi impregnation method, which showed no detectable difference in the density of spines between the two genotypes in the CA1 subfield (data not shown). However, the morphology of the spines appeared to be altered and irregularly shaped spines (thin spines that cannot be categorized into mushroom, thin or stubby, e.g. branched spines) were increased in the NP<sup>-/-</sup> mice compared with the NP<sup>+/+</sup> mice in the CA1 subfield ( $2.4 \pm 0.4$  versus  $1.2 \pm 0.3$ ,  $P < 0.05$ ). We therefore conclude that neuropsin deficiency induced the abnormal morphology of the spines.

### Discussion

L1 is known as a major adhesion molecule of growth cones, involved in elongation, fasciculation and pathfinding (Schachner, 1989; Burden-Gulley et al., 1995). However, its roles in mature synaptic functions such as synaptic plasticity are poorly understood. Previous immunohistochemical studies failed to observe L1ir in synapses of adult mice (Persohn and Schachner, 1990), although antibodies to L1 and a recombinant L1 fragment efficiently inhibited mature synaptic functions such as LTP (Luthi et al., 1994). Here and in other recent studies, we found L1 immunoreactivity in presynaptic immature boutons in the mature hippocampus (Matsumoto-Miyai et al., 2003; Munakata et al., 2003), probably resulting from a thorough examination of all the layers of the stratum radiatum and the use of two kinds of L1-specific antibodies, which appear to have minimum steric interference between the epitopic intracellular domain and other molecules. Additionally, close association of L1 with the presynaptic membrane could be observed using colloidal gold as an immunoreactivity marker.

Recently, several investigators have described orphan boutons as another functioning synapse-like structure (Shepherd and Harris, 1998; Krueger et al., 2003). Many orphan vesicle-release sites lacking postsynaptic specialization are present in cultured hippocampal neurons, and are proposed to be initiation sites of synaptogenesis (Krueger et al., 2003). On the other hand, the size of the spine and PSD correlated directly with the amount of AMPA receptor (Harris and

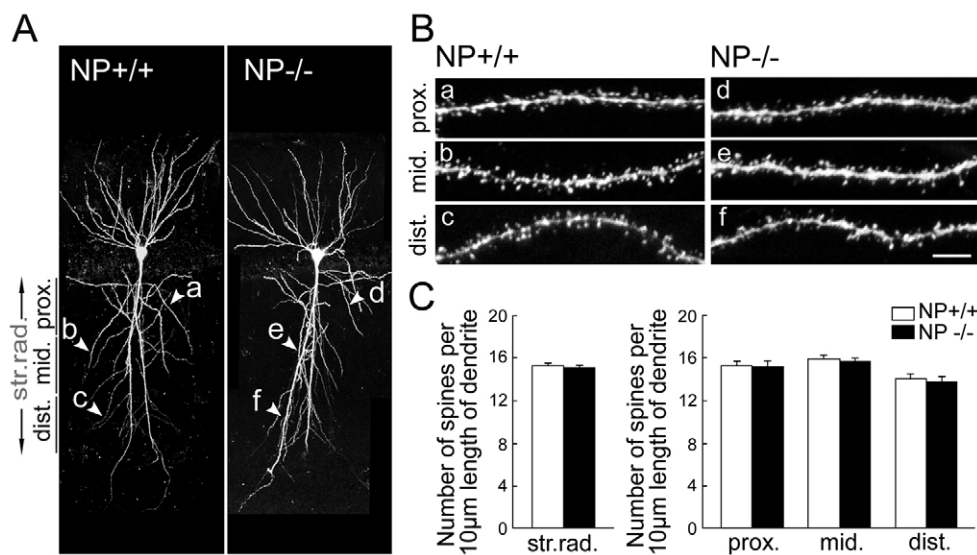


**Fig. 6.** Injection of recombinant active neuropsin into NP<sup>-/-</sup> mice restores L1ir synapses to NP<sup>+/+</sup> levels. (A) Fluorescent microscopic photograph represent spread of recombinant actneuropsin in the hippocampus. Injection of FITC-conjugated recombinant actneuropsin reaches the observation site of the stratum radiatum. (B) Number (left) and percentage (right) of L1ir synapses one day after injection of ACSF or active neuropsin (actNP) into NP<sup>-/-</sup> mice. The number and percentage of L1ir synapses were significantly decreased (actNP, closed bars; \* $P < 0.05$ ) to the levels in untreated wild-type mice (untreated, open bars). There was no significant difference between the treated NP<sup>-/-</sup> mice and untreated wild-type mice (number,  $P > 0.24$ ; percentage,  $P > 0.08$ ). \*\* $P < 0.001$ .

**Fig. 7.** Density of spines is the same in NP<sup>+/+</sup> and NP<sup>-/-</sup> mice.

(A) Fluorescence images of hippocampal CA1 pyramidal neurons of NP<sup>+/+</sup> (left) and NP<sup>-/-</sup> mice (right) after injection of Lucifer Yellow.

Arrowheads denote areas enlarged in B: proximal (prox.) (a and d), middle (mid.) (b and e) and distal (dist.) (c and f). (B) Spine morphology of NP<sup>+/+</sup> (left) and NP<sup>-/-</sup> mice in prox. (a and d), mid. (b and e) and dist. (c and f) regions. Images are from the regions marked by the respective arrowheads in A. Bar, 5  $\mu$ m. (C) Spines were counted in NP<sup>+/+</sup> (open bars) and NP<sup>-/-</sup> (closed bars) mice. The left graph shows the density of spines in the stratum radiatum, and the right graph shows that in each region according to the distance from the CA1 soma. There was no difference in the density of spines between NP<sup>+/+</sup> and NP<sup>-/-</sup> mice.

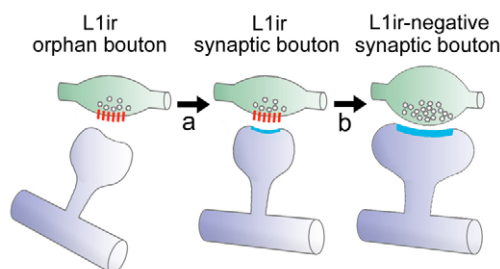


Stevens, 1989; Isaac et al., 1995; Liao et al., 1995; Schikorski and Stevens, 1999; Takumi et al., 1999), whereas a thin spine is thought to be an immature type of synapse that occurs during conversion to the mature mushroom spine (Tashiro and Yuste, 2004). Another report shows that dendritic spines are inducible by an extracellular factor at the cell surface as part of a receptor-ligand interaction (Passafaro et al., 2003). Thus, synaptogenesis may start at the onset of communication between orphan boutons and immature spines, followed by the maturation of synapse. Synapse formation could therefore be divided into two phases, as shown schematically in Fig. 8: an early phase where orphan boutons make contact with small spines (Fig. 8a), and a late phase where small synapses grow into large synapses (Fig. 8b). Between the early and the late phase, a high-frequency stimulus that elicits LTP may induce an NMDA-receptor-dependent signaling cascade; these steps are involved in the formation of memory, as shown by a number of studies (for a review, see Collingridge and Bliss, 1995).

In this study, we found orphan boutons in the CA1 stratum radiatum, and surprisingly, a considerable percentage of them (64–84%) were L1ir. On the other hand, most synaptic boutons were L1ir-negative (Table 1); in addition, the number of L1ir synaptic vesicles was low and the morphology and size of spines contacted with L1ir boutons were thin and small. These data strongly suggest that L1 becomes localized at the beginning of, or immediately after, synaptogenesis, and that L1ir presynaptic boutons (including orphan boutons) are an immature type of transmission machinery. In neuropsin-deficient mice, both L1ir orphan and synaptic boutons were observed in larger numbers, although the total number of synapses was decreased. The data suggest that maturation of synaptic structure was inhibited by neuropsin deficiency. Since neuropsin deficiency caused an accumulation of L1ir orphan and synaptic boutons, neuropsin is likely to be involved in both phases; formation (a) and maturation (b) synapses (Fig. 8). This neuropsin-related synaptogenesis/maturation may be important for neural plasticity, including learning and memory

because our previous data have shown that chemically induced LTP causes a brief rise in neuropsin activity and breakdown of the L1 molecule in an NMDA-receptor-dependent manner (Matsumoto-Miyai et al., 2003). Thus, both high-frequency stimuli and activation of neuropsin may be required for neuropsin-dependent synaptogenesis/maturation of L1ir boutons.

In the present study, we found that L1ir boutons accumulated and the total number of synapses decreased in NP<sup>-/-</sup> mice. The basal synaptic transmission of Schaffer-collateral CA1 synapses appears to be reduced in NP<sup>-/-</sup> mice (supplementary material Fig. S1), implying that functional synapses decrease in the NP<sup>-/-</sup> hippocampus. Electrophysiological and quantitative immunoEM studies both indicated that mature and functional synapses decrease in the mutant, and therefore neuropsin may mediate the conversion of immature synapses to mature synapses. The rather late expression of hippocampal neuropsin mRNA during ontogeny indicates that neuropsin has a major function in the adult stage (Suzuki et al., 1995). As shown by



**Fig. 8.** Diagrammatic model of synapse conversion. Formation of synapses is divided into two phases, an early phase where L1ir orphan boutons make contact with small spines (a) and a late phase where small synapses containing L1 grow into large synapses lacking L1 (b). Neuropsin is considered to be involved in both phases. Presynaptic boutons are shown in pale green, spines in pale blue, PSD is bright blue, L1 in red, and synaptic vesicles as circles.



the experiment in which recombinant actNP was microinjected into neuropsin-deficient mice (Fig. 6), neuropsin retained the ability to convert boutons to the mature type. When actNP was injected into the CA1 region of the mature hippocampus of 8-week-old NP<sup>-/-</sup> mice, the number of L1ir synaptic boutons recovered to the level seen in the wild-type. Thus, conversion of L1ir synaptic boutons to L1ir-negative synapses may occur continuously in the adult hippocampus, presumably driven by an NMDA-dependent transient activation of proneuropsin (Matsumoto-Miyai et al., 2003). In the neuropsin-deficient mouse hippocampus, hypertrophied L1ir orphan and synaptic boutons were often observed. This may be caused by the continuous growth of synapses, without modification or stabilization of L1ir immature synapses by neuropsin. Activity-dependent synaptic conversion from immature to mature by neuropsin may trigger a chain reaction of a variety of LTP-related molecular events, such as the insertion of AMPA receptors, and molecular changes in adhesion molecules.

In this study, negative effects of neuropsin deficiency were observed particularly in the proximal region from CA1 soma, although every layer in the stratum radiatum showed the same tendency. It has been shown recently that synaptic character varies according to distance from the soma, to differences in the distribution pattern of spines and A-type K<sup>+</sup> channels, and to synaptic responsiveness (Hoffman et al., 1997; Megias et al., 2001; Oray et al., 2004). Our present data and these earlier observations indicate that synaptic character is not homogeneous over the stratum radiatum, and that neuropsin may be involved in activity-dependent synaptogenesis/maturation, most effectively in the proximal one-third of the stratum radiatum.

## Materials and Methods

### Animals

Production of NP<sup>-/-</sup> mice was described by Hirata et al. (Hirata et al., 2001). The mice were backcrossed six times with strain C57BL/6J. Mice were maintained according to guidelines of the Nara Institute of Science and Technology, and the study was approved by the institutional animal care and use committee.

### Tissue preparation

Male NP<sup>+/+</sup> and NP<sup>-/-</sup> (8-week-old) mice were deeply anesthetized with an intraperitoneal injection of 10% ethylcarbamate (10 µl/g body weight) and perfused through the ascending aorta with 50 ml saline and then 100 ml fixative [0.1 M phosphate-buffered saline (PBS), pH 7.4, 4% paraformaldehyde, 0.05% glutaraldehyde]. Brains were removed and postfixed overnight in the same fixative at 4°C and coronal-sectioned with a Microslicer (Dosaka EM, Kyoto, Japan) at a thickness of 30 µm. Tissue preparations and observations were all performed at the same front-caudal brain level (bregma -2.30 mm) of the mouse brain atlas of Franklin and Paxinos (Franklin and Paxinos, 1996).

### Immunohistochemistry for light microscopy

The immunohistochemistry of L1 was as described previously (Munakata et al., 2003). Briefly, the tissue sections were pre-incubated in PBS containing 5% bovine serum albumin (BSA) (Sigma, St Louis, MO) for 1 hour. The sections were incubated in anti-C-terminal L1 antiserum (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200). For detection of anti-L1 antibody, biotinylated anti-goat immunoglobulin G (IgG) secondary antibody was used (Vector Laboratories, Burlingame, CA; 1:1000). Bound antibody was detected by staining for 2 hours with the ABC Vectastain kit (Vector Laboratories, Burlingame, CA) and then for 5 minutes with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 50 mM Tris-HCl, pH 7.5, containing 0.01% H<sub>2</sub>O<sub>2</sub>. The sections were observed under a Zeiss Axioplan 2 microscope, and images of each section were captured with a C4742-95 digital camera (Hamamatsu Photonics, Hamamatsu, Japan) and analyzed with Win Roof software (Mitani, Fukui, Japan).

### Pre-embedding immunohistochemistry and quantitative electron microscopy

Sections for EM were postfixed with 1% OsO<sub>4</sub> in 0.1 M phosphate buffer (pH 7.4) for 1 hour at 4°C, and dehydrated in a series of solutions containing ethanol and propylene oxide. The sections were mounted with Epon 812 on a siliconized

glass slide. After light microscopic observation, equivalent hippocampal regions of NP<sup>+/+</sup> and NP<sup>-/-</sup> mice were cut out and sectioned with a Reinhardt ultramicrotome (Heidelberg, Germany) at a thickness of 80 nm. The ultrathin sections were stained with lead citrate and observed using a Hitachi H-7100 EM (Tokyo, Japan), and images were acquired using a Polaroid film scanner (Tokyo, Japan). The number of L1-immunoreactive (L1ir) and L1ir-negative boutons was counted in a fixed area (13.0×73.2 µm). Three consecutive sections were observed to determine whether boutons were orphan or synaptic for statistical analysis (Hirata et al., 2001).

### Post-embedding immunohistochemistry

Small tissue blocks (300 µm) from the CA1 region of the hippocampus were subjected to freeze-substitution and low-temperature embedding in Lowicryl K4M (Polysciences, Eppelheim, Germany). Ultrathin sections (80 nm) were mounted on Formvar-coated grids and processed for immunogold histochemistry. Sections were immersed in 3% H<sub>2</sub>O<sub>2</sub> for 3 minutes and incubated at room temperature for 10 minutes in 0.1% sodium borohydride and 50 mM glycine in 5 mM Tris-HCl containing 50 mM NaCl and 0.1% Triton X-100 (TBST), followed by incubation in 1% bovine serum albumin (BSA) in TBST, then for 12–16 hours in mixtures of goat antibodies to L1 diluted 1:100 in TBST containing 1% BSA. For detection of anti-L1 antibody, biotinylated anti-goat IgG secondary antibody was used (1:200). Then sections were placed in streptavidin-coupled 10-nm gold particles (EM-STP10; British BioCell International, Cardiff, UK) diluted 1:25 in TBST containing 1% BSA and polyethylene glycol (5 mg/ml) for 2 hours.

### Statistical analysis

To count the number of synapses and orphan boutons and measuring spines and PSDs, 20 mice (10 NP<sup>+/+</sup> and 10 NP<sup>-/-</sup>) were used. For counting synapses after injection of active neuropsin into NP<sup>-/-</sup> mice, three animals were used and an investigator blind to the conditions performed all analyses. Mean numbers and size of L1ir and L1ir-negative boutons in NP<sup>+/+</sup> and NP<sup>-/-</sup> mice were compared using unpaired two-tailed Student's *t*-tests, and distribution analysis was performed using the Kolmogorov-Smirnov test.

### In situ hybridization

In situ hybridization of L1 was described previously (Horinouchi et al., 2005). Briefly, 16-µm sections were fixed with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) (PB), permeabilized with 10 µg/ml proteinase K in 50 mM Tris-HCl (pH 7.5) and 5 mM EDTA and acetylated in 0.1 M triethanolamine buffer containing 0.225% acetic anhydride. After dehydration, the sections were hybridized with 10<sup>7</sup> cpm/ml <sup>35</sup>S-labeled cRNA probe at 55°C for 16 hours, washed, and then treated with RNaseA. After dehydration, the sections were dipped in Kodak NBT-2 emulsion (Rochester, NY) and exposed for 1 month.

### 3D reconstruction of images from serial ultrathin sections

Entire synapses including spines and synaptic boutons were reconstructed from image stacks of 20 serial sections. Seventeen randomly selected synapses of L1ir and L1ir-negative boutons from NP<sup>-/-</sup> and NP<sup>+/+</sup> mice were reconstructed. Digital images were aligned using IGL Trace software (developed by John C. Fiala, Boston University, Boston, MA). Contours of axons, dendrites, PSDs and boutons were displayed three-dimensionally with GLView version 4.30 software (Bitmanagement Software, Germany).

### Western blot analysis

Hippocampi were removed from 6 NP<sup>-/-</sup> and 6 NP<sup>+/+</sup> (C57BL/6J) mouse brains. Each hippocampal organ was homogenized in ice-cold lysis buffer containing 20 mM Tris-HCl, 0.32 M sucrose, 1 mM EDTA, 1 mM sodium orthovanadate and protease inhibitor cocktail (Sigma, St Louis, MO). The homogenates were centrifuged for 10 minutes at 900 *g* to remove debris, nuclei, etc. The supernatant was treated with Triton X-100 at a final concentration of 1%. Aliquots of the proteins were subjected to SDS-PAGE and transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA). Membrane were pretreated with 5% skimmed milk and 0.1% Tween 20 in TBS (TBST), pH 7.4. The membrane was incubated with anti-C-terminal L1 mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA; 1:2000) or anti-actin monoclonal IgG (Chemicon, Temecula, CA; 1:1000). After washing in TBST, the blots were incubated with alkaline-phosphatase-conjugated rabbit anti-mouse IgG antibody. Immunoreactivity was detected with Immuno-Star substrate (Bio-Rad Laboratories, Hercules, CA) on X-ray film according to the manufacturer's instructions. Band density was quantified with the Scion Image software (Scion, Frederick, MD).

### Intracellular injections of Lucifer Yellow

Mice were anesthetized with diethyl ether, and the brains were removed into modified Ringer solution (46.8 mM sucrose, 0.5 mM KCl, 0.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 5.2 mM NaHCO<sub>3</sub>, 2.2 mM glucose, 2 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub>) keeping it oxygenated (95% O<sub>2</sub>/5% CO<sub>2</sub>) for 5 minutes. The brain was sectioned on a vibratome (300 µm thickness), placed in oxygenated ACSF solution for 1–2 hours at 32°C, and then transferred to cold 4% paraformaldehyde in 0.1 M PB for 45



minutes. Lucifer Yellow was injected with an Eppendorf Microinjector 5242 (Hamburg, Germany) by applying negative current pulses (1.5 nA) for 10 minutes. Sections with successfully filled neurons were transferred into cold 4% paraformaldehyde in 0.1 M PB, postfixed overnight, and then photographed under a Nikon Eclipse E600 microscope (Tokyo, Japan) to observe dendritic arbors and spines of CA1 pyramidal neurons.

### Injection of active neuropsin

Three 8-week-old NP<sup>-/-</sup> mice were anesthetized with 10% ethyl carbamate (1.25 mg/kg), placed in a stereotaxic apparatus, and injected with recombinant active neuropsin using a 5 µl Hamilton (Reno, NV) syringe over 10 minutes. Stereotaxic coordinates relative to bregma were 1.34 mm posterior and 1.20 mm lateral, and the tip of the infusion cannula (26 gauge) was located in the stratum radiatum of the CA1 field. The recombinant actNP was produced as described (Shimizu et al., 1998; Komai et al., 2000) and diluted with artificial cerebrospinal fluid (ACSF; 127 mM NaCl, 1.6 mM KCl, 1.24 mM KH<sub>2</sub>PO<sub>4</sub>, 1.3 mM MgSO<sub>4</sub>, 2.4 mM CaCl<sub>2</sub>, 26 mM NaHCO<sub>3</sub>, 10 mM glucose). FITC-conjugated recombinant actneuropsin was injected to observe the spread area of recombinant actneuropsin over the hippocampal sublayers. The dose of recombinant actNP used in this study (0.8 µl, 1.8 mU/ml) was the optimum concentration leading to an increase of the Schaffer-collateral path-evoked fEPSP slope (Tamura et al., 2006).

### Electrophysiology

Male NP<sup>+/+</sup>, NP<sup>+/-</sup> and NP<sup>-/-</sup> mice were anaesthetized with urethane (1.25 g/kg; i.p.) and placed in a stereotaxic frame with the skull horizontal. Rectal temperature was maintained at 37°C using a heated jacket pad (BWT-100, Bio Research Center, Nagoya, Japan). Extracellular field recording was carried out as previously described (Matsumoto-Miyai et al., 2003). A bipolar stainless-steel electrode (InterMedical, Tokyo, Japan) was used for stimulation in the Schaffer-collateral pathway (2.46 mm posterior and 2.30 mm lateral to bregma). Field excitatory postsynaptic potentials (fEPSPs) were recorded with a monopolar tungsten electrode (InterMedical) placed in the stratum radiatum of the CA1 region (2.46 mm posterior and 2.0 mm lateral to bregma). After a stable response was reached, I-O curves were obtained over the range 40–600 µA.

We thank Vance Lemmon of University of Miami School of Medicine for critically reading the manuscript and Ian R. L. Smith of Nara Institute of Science and Technology for correcting the manuscript. We also thank Yoshinori Kawai of the Jikei University School of Medicine and Megumi Iwano of Nara Institute of Science and Technology for useful advice on electronmicroscopic technique. This work is supported in part by a Grant-in-Aid for 21st Century COE Research from Ministry of Education, Culture, Sports, Science and Technology.

### References

- Benson, D. L., Schnapp, L. M., Shapiro, L. and Huntley, G. W. (2000). Making memories stick: cell-adhesion molecules in synaptic plasticity. *Trends Cell Biol.* **10**, 473–482.
- Burden-Gulley, S. M., Payne, H. R. and Lemmon, V. (1995). Growth cones are actively influenced by substrate-bound adhesion molecules. *J. Neurosci.* **15**, 4370–4381.
- Collingridge, G. L. and Bliss, T. V. (1995). Memories of NMDA receptors and LTP. *Trends Neurosci.* **18**, 54–56.
- Fiala, J. C. and Harris, K. M. (1999). Dendritic structure. In *Dendrite* (ed. G. Stuart, N. Spruston and M. Hausser), pp. 1–34. New York: Oxford University Press.
- Franklin, K. B. J. and Paxinos, G. (1996). *The Mouse Brain*. San Diego: Academic Press.
- Harris, K. M. and Stevens, J. K. (1989). Dendritic spines of CA 1 pyramidal cells in the rat hippocampus: serial electron microscopy with reference to their biophysical characteristics. *J. Neurosci.* **9**, 2982–2997.
- Hirata, A., Yoshida, S., Inoue, N., Matsumoto-Miyai, K., Ninomiya, A., Taniguchi, M., Matsuyama, T., Kato, K., Iizasa, H., Kataoka, Y. et al. (2001). Abnormalities of synapses and neurons in the hippocampus of neuropsin-deficient mice. *Mol. Cell Neurosci.* **17**, 600–610.
- Hoffman, D. A., Magee, J. C., Colbert, C. M. and Johnston, D. (1997). K<sup>+</sup> channel regulation of signal propagation in dendrites of hippocampal pyramidal neurons. *Nature* **387**, 869–875.
- Horinouchi, K., Nakamura, Y., Yamanaka, H., Watabe, T. and Shiosaka, S. (2005). Distribution of L1-cam mRNA in the adult mouse brain: in situ hybridization and northern blot analyses. *J. Comp. Neurol.* **482**, 386–404.
- Isaac, J. T., Nicoll, R. A. and Malenka, R. C. (1995). Evidence for silent synapses: implications for the expression of LTP. *Neuron* **15**, 427–434.
- Komai, S., Matsuyama, T., Matsumoto, K., Kato, K., Kobayashi, M., Imamura, K., Yoshida, S., Ugawa, S. and Shiosaka, S. (2000). Neuropsin regulates an early phase of schaffer-collateral long-term potentiation in the murine hippocampus. *Eur. J. Neurosci.* **12**, 1479–1486.
- Krueger, S. R., Kolar, A. and Fitzsimonds, R. M. (2003). The presynaptic release apparatus is functional in the absence of dendritic contact and highly mobile within isolated axons. *Neuron* **40**, 945–957.
- Liao, D., Hessler, N. A. and Malinow, R. (1995). Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. *Nature* **375**, 400–404.
- Luthi, A., Laurent, J. P., Figurov, A., Muller, D. and Schachner, M. (1994). Hippocampal long-term potentiation and neural cell adhesion molecules L1 and NCAM. *Nature* **372**, 777–779.
- Matsumoto-Miyai, K., Ninomiya, A., Yamasaki, H., Tamura, H., Nakamura, Y. and Shiosaka, S. (2003). NMDA-dependent proteolysis of presynaptic adhesion molecule L1 in the hippocampus by neuropsin. *J. Neurosci.* **23**, 7727–7736.
- Matsuzaki, M., Honkura, N., Ellis-Davies, G. C. and Kasai, H. (2004). Structural basis of long-term potentiation in single dendritic spines. *Nature* **429**, 761–766.
- Megias, M., Emri, Z., Freund, T. F. and Gulyas, A. I. (2001). Total number and distribution of inhibitory and excitatory synapses on hippocampal CA1 pyramidal cells. *Neuroscience* **102**, 527–540.
- Momota, Y., Yoshida, S., Ito, J., Shibata, M., Kato, K., Sakurai, K., Matsumoto, K. and Shiosaka, S. (1998). Blockade of neuropsin, a serine protease, ameliorates kindling epilepsy. *Eur. J. Neurosci.* **10**, 760–764.
- Munakata, H., Nakamura, Y., Matsumoto-Miyai, K., Itoh, K., Yamasaki, H. and Shiosaka, S. (2003). Distribution and densitometry mapping of L1-CAM Immunoreactivity in the adult mouse brain—light microscopic observation. *BMC Neurosci.* **4**, 7.
- Oka, T., Hakoshima, T., Itakura, M., Yamamori, S., Takahashi, M., Hashimoto, Y., Shiosaka, S. and Kato, K. (2002). Role of loop structures of neuropsin in the activity of serine protease and regulated secretion. *J. Biol. Chem.* **277**, 14724–14730.
- Okabe, A., Momota, Y., Yoshida, S., Hirata, A., Ito, J., Nishino, H. and Shiosaka, S. (1996). Kindling induces neuropsin mRNA in the mouse brain. *Brain Res.* **728**, 116–120.
- Oray, S., Majewska, A. and Sur, M. (2004). Dendritic spine dynamics are regulated by monocular deprivation and extracellular matrix degradation. *Neuron* **44**, 1021–1030.
- Passafium, M., Nakagawa, T., Sala, C. and Sheng, M. (2003). Induction of dendritic spines by an extracellular domain of AMPA receptor subunit GluR2. *Nature* **424**, 677–681.
- Persohn, E. and Schachner, M. (1990). Immunohistological localization of the neural adhesion molecules L1 and N-CAM in the developing hippocampus of the mouse. *J. Neurocytol.* **19**, 807–819.
- Schachner, M. (1989). Families of neural adhesion molecules. *Ciba Found. Symp.* **145**, 156–169.
- Schikorski, T. and Stevens, C. F. (1999). Quantitative fine-structural analysis of olfactory cortical synapses. *Proc. Natl. Acad. Sci. USA* **96**, 4107–4112.
- Shepherd, G. M. and Harris, K. M. (1998). Three-dimensional structure and composition of CA3→CA1 axons in rat hippocampal slices: implications for presynaptic connectivity and compartmentalization. *J. Neurosci.* **18**, 8300–8310.
- Shimizu, C., Yoshida, S., Shibata, M., Kato, K., Momota, Y., Matsumoto, K., Shiosaka, T., Midorikawa, R., Kamachi, T., Kawabe, A. et al. (1998). Characterization of recombinant and brain neuropsin, a plasticity-related serine protease. *J. Biol. Chem.* **273**, 11189–11196.
- Shiosaka, S. and Yoshida, S. (2000). Synaptic microenvironments—structural plasticity, adhesion molecules, proteases and their inhibitors. *Neurosci. Res.* **37**, 85–89.
- Suzuki, J., Yoshida, S., Chen, Z. L., Momota, Y., Kato, K., Hirata, A. and Shiosaka, S. (1995). Ontogeny of neuropsin mRNA expression in the mouse brain. *Neurosci. Res.* **23**, 345–351.
- Takumi, Y., Ramirez-Leon, V., Laake, P., Rinivik, E. and Ottersen, O. P. (1999). Different modes of expression of AMPA and NMDA receptors in hippocampal synapses. *Nat. Neurosci.* **2**, 618–624.
- Tamura, H., Ishikawa, Y., Hino, N., Maeda, M., Yoshida, S., Kaku, S. and Shiosaka, S. (2006). Neuropsin is essential for early processes of memory acquisition and Schaffer collateral long-term potentiation in adult mouse hippocampus in vivo. *J. Physiol.* **570**, 541–551.
- Tashiro, A. and Yuste, R. (2004). Regulation of dendritic spine motility and stability by Rac1 and Rho kinase: evidence for two forms of spine motility. *Mol. Cell Neurosci.* **26**, 429–440.