NR2B tyrosine phosphorylation modulates fear learning as well as amygdaloid synaptic plasticity

Takanobu Nakazawa^{1,6}, Shoji Komai^{2,6},
Ayako M Watabe^{2,6}, Yuji Kiyama²,
Masahiro Fukaya⁴, Fumiko Arima-Yoshida²,
Reiko Horai³, Katsuko Sudo³, Kazumi
Ebine³, Mina Delawary¹, June Goto¹,
Hisashi Umemori¹, Tohru Tezuka¹,
Yoichiro Iwakura³, Masahiko Watanabe⁴,
Tadashi Yamamoto^{1,*} and
Toshiya Manabe^{2,5,*}

¹Division of Oncology, Department of Cancer Biology, University of Tokyo, Tokyo, Japan; ²Division of Neuronal Network, Department of Basic Medical Sciences, University of Tokyo, Tokyo, Japan; ³Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan; ⁴Department of Anatomy, Hokkaido University School of Medicine, Sapporo, Japan and ⁵CREST, Japan Science and Technology Agency, Tokyo, Japan

Phosphorylation of neural proteins in response to a diverse array of external stimuli is one of the main mechanisms underlying dynamic changes in neural circuitry. The NR2B subunit of the NMDA receptor is tyrosine-phosphorylated in the brain, with Tyr-1472 its major phosphorylation site. Here, we generate mice with a knockin mutation of the Tyr-1472 site to phenylalanine (Y1472F) and show that Tyr-1472 phosphorylation is essential for fear learning and amygdaloid synaptic plasticity. The knockin mice show impaired fear-related learning and reduced amygdaloid long-term potentiation. NMDA receptor-mediated CaMKII signaling is impaired in YF/YF mice. Electron microscopic analyses reveal that the Y1472F mutant of the NR2B subunit shows improper localization at synapses in the amygdala. We thus identify Tyr-1472 phosphorylation as a key mediator of fear learning and amygdaloid synaptic plasticity.

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*Corresponding authors. T Yamamoto, Division of Oncology, Department of Cancer Biology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan. Tel.: +81 3 5449 5301; Fax: +81 3 5449 5413; E-mail: tyamamoto@ims.u-tokyo.ac.jp or T Manabe, Division of Neuronal Network, Department of Basic Medical Sciences, University of Tokyo, Tokyo 108-8639, Japan. Tel.: +81 3 5449 5799; Fax: +81 3 5449 5794; E-mail: tmanabe-ky@umin.ac.jp

⁶These authors contributed equally to this work

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Introduction

The *N*-methyl-D-aspartate subtype of ionotropic glutamate receptor (NMDAR) is crucial for development, synaptic plasticity and neuronal excitotoxicity (Choi, 1988; McDonald and Johnston, 1990; Collingridge and Bliss, 1995). Long-term potentiation (LTP) of excitatory synaptic transmission is a candidate for the cellular mechanism underlying neural plasticity, such as learning and memory (Bliss and Collingridge, 1993). The NMDAR is composed of the NR1 (GluR ζ) and NR2 (GluR ϵ) subunits. The NR1 subunit is essential for the function of NMDAR channels, whereas NR2 subunits (NR2A (GluR ϵ 1), NR2B (GluR ϵ 2), NR2C (GluR ϵ 3) and NR2D (GluR ϵ 4)) determine the characteristics of NMDAR channels by forming different heteromeric configurations with the NR1 subunit (Monyer *et al*, 1994; Cull-Candy *et al*, 2001). The NMDAR complex is composed of NMDAR subunits and a number of associated regulatory proteins, including scaffolding proteins, cytoskeletal proteins, protein kinases and protein phosphatases (Kornau *et al*, 1997; Husi *et al*, 2000; Scannevin and Huganir, 2000; Sheng and Kim, 2002).

Recent studies have shown that NMDARs are subject to rapid activity-dependent redistribution at synapses (Carroll and Zukin, 2002; Wenthold *et al*, 2003). The NMDAR moves laterally between synaptic and extrasynaptic domains. LTP promotes rapid expression of NMDARs on the cell surface at hippocampal CA1 synapses. Conversely, long-term depression (LTD) correlates with a decrease in NMDAR protein levels in CA1 synaptoneurosome (Carroll and Zukin, 2002; Wenthold *et al*, 2003). Moreover, the NMDAR is internalized by clathrin-mediated endocytosis from synaptic sites in a use-dependent manner (Roche *et al*, 2001; Vissel *et al*, 2001), which is thought to contribute to several forms of synaptic plasticity.

The amygdala is a group of spatially contiguous and anatomically interconnected nuclei located within the rostral pole of the temporal lobe of mammals. Accumulated evidence indicates that the amygdala is a crucial brain structure for the expression of fear memory (Davis, 1997; Fendt and Fanselow, 1999; Maren, 1999; LeDoux, 2000; Chapman *et al*, 2003; Sah *et al*, 2003). LTP is observed in both thalamo-amygdala and cortico-amygdala projections *in vivo* (Maren, 1999; LeDoux, 2000; Chapman *et al*, 2003). Given that LTP occurs at synapses receiving conditioned stimulus and that fear conditioning induces associative LTP-like changes (Maren, 1999; LeDoux, 2000; Chapman *et al*, 2003), the LTP is a candidate mechanism for memory storage during fear memory formation. Intra-amygdala blockade of NMDARs disrupts both the acquisition of fear memory and LTP, indicating that NMDARs in the amygdala are necessary for fear memory formation (Gewirtz and Davis, 1997; Lee and Kim, 1998; LeDoux, 2000; Fendt, 2001; Rodrigues *et al*, 2001; Bauer *et al*, 2002).

NR2 subunits have an unusually long C-terminal tail extended into the cytoplasm (Cull-Candy *et al*, 2001), which

is likely to play essential roles in their regulation including receptor localization, receptor channel activity and downstream signaling via NMDAR-associated proteins (Kornau *et al*, 1997; Husi *et al*, 2000; Scannevin and Huganir, 2000; Sheng and Kim, 2002). Several proteins including scaffolding proteins, cytoskeletal proteins, signaling proteins, protein kinases and protein phosphatases interact directly with NR2B subunits (Kornau *et al*, 1997; Husi *et al*, 2000; Scannevin and Huganir, 2000; Sheng and Kim, 2002).

The NR2B subunit is a major tyrosine-phosphorylated protein (Moon *et al*, 1994). We have identified Tyr-1472 of the NR2B subunit as the main phosphorylation site *in vitro* and have shown that this tyrosine is phosphorylated by Fyn, a member of the Src family kinases (Nakazawa *et al*, 2001). The level of Tyr-1472 phosphorylation is increased after induction of LTP in the hippocampus, suggesting that Fyn-mediated phosphorylation of Tyr-1472 is involved in synaptic plasticity (Nakazawa *et al*, 2001). Tyr-1472 phosphorylation is also implicated in transient ischemia, lithium protection against excitotoxicity and ethanol inhibition of NMDAR-mediated responses (Salter and Kalia, 2004). Src and Fyn upregulate the current through recombinant NR1-NR2A channels, but not NR1-NR2B channels in HEK293 cells (Köhr and Seeburg, 1996). Thus, Tyr-1472 phosphorylation may not regulate NMDAR channel properties but rather regulate NMDAR localization or initiation of downstream signaling pathways. Such action may be triggered by provid-

ing binding sites for signaling proteins through SH2 or phospho-tyrosine-binding domains. However, the precise roles of Tyr-1472 phosphorylation *in vivo* remain unclear.

Here, we report analyses of knockin mice in which Tyr-1472 of the NR2B subunit is mutated to phenylalanine to prevent phosphorylation of this site *in vivo*. We find that the lack of Tyr-1472 phosphorylation results in poor fear-related learning, impaired amygdaloid synaptic plasticity, and improper NMDAR localization at synapses.

Results

Generation of NR2B Y1472F knockin mice

To examine the physiological significance of Tyr-1472 phosphorylation of the NR2B subunit, we generated mutant mice lacking the Tyr-1472 phosphorylation site by substituting Tyr-1472 (Y) with Phe-1472 (F) by a knockin technique (Figure 1). The success of these procedures was confirmed by Southern blot and PCR analysis (Figure 1A–C and data not shown). We confirmed the absence of Tyr-1472 phosphorylation in homozygous knockin mice (YF/YF mice) by immunoblot analysis using the anti-pY1472 antibody that specifically recognizes the NR2B subunit phosphorylated at Tyr-1472 (Nakazawa *et al*, 2001; Figure 1C, top panel). The expression level of the NR2B subunit in YF/YF mice was virtually identical to that in wild-type littermates (WT/WT mice), indicating that the targeted NR2B gene expressed the NR2B

Figure 1 Generation of mice with a mutation of the NR2B tyrosine phosphorylation site. (A) Schematic representations of the structures of WT, targeting vector, and targeted and floxed-targeted NR2B alleles with relevant restriction sites. The probe for Southern blotting is indicated. pBKS, pBluescriptIIKS plasmid; DTA, diphtheria toxin gene; neo, neomycin-resistance gene. (B) The Southern blot analysis of *HindIII*-digested genomic DNAs from WT/WT, WT/YF and YF/YF mice. (C) The absence of Tyr-1472 phosphorylation in homozygous YF/YF mice (upper). Equal amounts of amygdaloid lysates from WT/WT and YF/YF mice were probed with the anti-pY1472 antibody. (D) A reduced level of NR2B tyrosine-phosphorylation in YF/YF mice. Equal amounts of NR2B immunoprecipitates from amygdaloid lysates of WT/WT and YF/YF mice were probed with the anti-pY (4G10) mAb. A representative blot is shown in the inset. All data are presented as the mean $\pm$ s.e.m. (Figures 1–7 and Supplementary Figures 1–7). **P* < 0.001, Student's *t*-test.

protein at normal levels (WT/WT, $100 \pm 7\%$, $n = 5$ from five mice; YF/YF, $102 \pm 5\%$, $n = 5$ from five mice; $P > 0.9$, Student's *t*-test) (Figure 1C, bottom panel).

YF/YF mice appeared healthy with general behaviors (data not shown). Histological analysis with Nissl-stained coronal sections of CNS structures from YF/YF mice did not show any gross abnormalities in cytoarchitecture (data not shown). On the basis of the observed behavioral and electrophysiological phenotypes described below, we analyzed the amygdala in detail. In YF/YF mice, the levels of NR2B tyrosine phosphorylation in the amygdala (WT/WT, $100 \pm 11\%$, $n = 4$ from four mice; YF/YF, $21 \pm 5\%$, $n = 4$ from four mice; $P < 0.001$, Student's *t*-test), cerebral cortex and hippocampus were significantly reduced in comparison with those of WT/WT mice, indicating that Tyr-1472 was the main phosphorylation site *in vivo* (Figure 1D). The phosphorylation level of Tyr-1336, a minor phosphorylation site of NR2B (Nakazawa *et al*, 2001), was unchanged in YF/YF mice (Figure 1C, middle panel). These findings indicate that the lack of Tyr-1472 phosphorylation does not alter gross anatomy of the brain or expression of the NR2B subunit.

Impaired fear-related learning in YF/YF mice

We performed the battery of behavioral tests (Morris water maze test, contextual fear conditioning test, passive avoidance test and auditory fear conditioning test) for examining the learning ability of YF/YF mice. While we did not detect abnormalities in hippocampus-dependent learning ability of YF/YF mice (data not shown), YF/YF mice showed impaired fear-related learning in auditory fear conditioning test. Mice were given a single pairing of a tone (CS) and a footshock (US) on the conditioning day (Figure 2A). At 24 h after the conditioning, the mice were placed in a novel chamber for 3 min before the tone (CS) was delivered for 3 min. Before the tone was presented, both WT/WT and YF/YF mice displayed only weak freezing behavior in the novel chamber (WT/WT, $3.49 \pm 1.10\%$, $n = 17$; YF/YF, $5.54 \pm 2.01\%$, $n = 17$; $P > 0.3$, ANOVA) (Figure 2B and C). During the 1-min period immediately after delivery of the tone, WT/WT mice showed strong freezing responses ($41.6 \pm 4.8\%$, $n = 17$), whereas YF/YF mice showed significantly weaker responses ($23.1 \pm 6.5\%$, $n = 17$; $F_{(1,32)} = 5.23$, $P < 0.03$, ANOVA) (Figure 2B and C). This deficit could be caused by failure to acquire the CS-US association or by failure to stabilize the fear-related memory. To distinguish between these alternatives, we performed the behavioral test 1 h after the conditioning to assess the learning. In this test, YF/YF mice also showed much weaker freezing responses ($6.9 \pm 2.7\%$, $n = 10$) in comparison to WT/WT mice ($23.6 \pm 5.2\%$, $n = 20$; $F_{(1,28)} = 4.73$, $P < 0.04$, ANOVA) (Figure 2D), suggesting that YF/YF mice failed to acquire the CS-US association. Using visual conditioned stimuli, YF/YF mice also showed much weaker freezing responses in comparison to WT/WT mice (Supplementary Figure 1). Because pain sensitivity would affect freezing responses, we measured current thresholds for three reactions to the nociceptive shock; namely, flinch, vocalization and jump. There were no significant differences between WT/WT and YF/YF mice in pain thresholds for flinch (WT/WT, 0.07 ± 0.01 mA; YF/YF, 0.08 ± 0.01 mA; $F_{(1,15)} = 0.294$, $P > 0.5$, ANOVA), vocalization (WT/WT, 0.12 ± 0.01 mA; YF/YF, 0.12 ± 0.01 mA; $F_{(1,15)} = 0.340$, $P > 0.5$, ANOVA) and jump reactions (WT/WT,

Figure 2 Impairment of auditory fear conditioning in YF/YF mice. (A) Freezing responses of WT/WT and YF/YF mice in auditory fear conditioning on the conditioning day (at 3 min; WT/WT, 5.88 ± 1.43 , $n = 17$; YF/YF, 5.69 ± 1.41 , $n = 17$; $P > 0.9$, ANOVA). 170 s after mice were placed in the conditioning chamber, a tone was presented for 10 s (solid line); at the end of the tone mice were given a footshock (arrow). (B) Freezing responses 24 h after conditioning. At 3 min after mice were placed into a testing chamber with novel contexts, the tone was presented (solid line). WT/WT, $n = 17$; YF/YF, $n = 17$; $*P < 0.03$, ANOVA. (C, D) Summary of freezing responses 24 h after conditioning (C) and 1 h after conditioning (D). Freezing in the absence of the tone (Pre-CS) and in the presence of the tone (CS) was calculated as the mean freezing response from 2 to 3 min and from 3 to 4 min, respectively, after placement in the testing chamber. (24 h, $*P < 0.03$, ANOVA; 1 h, WT/WT, $n = 20$; YF/YF, $n = 10$; $*P < 0.04$, ANOVA).

0.29 ± 0.02 mA; YF/YF, 0.31 ± 0.03 mA; $F_{(1,15)} = 0.406$, $P > 0.5$, ANOVA). Together with our observations that YF/YF mice normally respond to the tone in acoustic startle response test (Supplementary Figure 2), these data suggest that fear-related learning is severely impaired in YF/YF mice.

Impaired synaptic plasticity of YF/YF mice

In the lateral nucleus of amygdala (LA), LTP can be induced at thalamic input synapses with a pairing protocol in which

weak presynaptic stimulation of thalamo-amygdala afferents that transmit CS information to the LA is presented concurrently with depolarization of the postsynaptic cell by current injection (Weisskopf *et al*, 1999). This form of LTP is essential for auditory fear conditioning in mice (LeDoux, 2000). We performed electrophysiological experiments in the LA of brain slices. Delivery of the conditioning stimulation to the thalamic inputs produced robust LTP in the LA of WT/WT mice ($155 \pm 14\%$ of baseline, $n = 17$), whereas LTP in YF/YF slices ($116 \pm 7\%$ of baseline, $n = 16$) was significantly smaller than that in WT/WT slices ($P < 0.02$, Student's *t*-test) (Figure 3A and B). The LTP was almost completely blocked by D-APV (Supplementary Figure 3A) or D-APV plus the L-type voltage-gated calcium channel (VGCC) antagonist nitrendipine (Supplementary Figure 3B), suggesting that the LTP was mostly NMDAR dependent. We found no significant differences ($P > 0.8$) between WT/WT (the ratio of integral of

the synaptic current during the first burst to that of the single excitatory postsynaptic potential (EPSC), 4.24 ± 0.24 , $n = 14$) and YF/YF (4.19 ± 0.32 , $n = 8$) mice in the temporal summation of NMDAR-mediated EPSCs elicited by the same stimulation protocol used for the LTP induction (Figure 3C). Thus, not only single NMDAR-mediated synaptic responses but also the multiple NMDAR-mediated synaptic responses (and presumably, Ca^{2+} influx into the postsynaptic cell) during the conditioning were unaltered by the mutation.

Increased phosphorylation of Tyr-1472 after tetanic stimulation in the amygdala

We then investigated whether the level of Tyr-1472 phosphorylation was enhanced after tetanic stimulation of thalamo-amygdala afferents (100 Hz for 1 s, repeated three times at 10-s intervals, three places). At 5 min after the stimulation, the level of Tyr-1472 phosphorylation was significantly enhanced

Figure 3 Impaired amygdaloid LTP in YF/YF mice. (A) The averaged time course of LTP in WT/WT ($n = 17$) and YF/YF ($n = 16$) mice. Maximal initial EPSP slopes were normalized in each experiment to the averaged maximal initial EPSP slope during the control period (-10 to 0 min). LTP was induced by 'pairing' at time 0 . Sample traces of EPSPs (average of 10 consecutive responses) in WT/WT and YF/YF mice recorded at the times indicated by the numbers are shown in the inset. (B) Summary of LTP (WT/WT, $n = 17$; YF/YF, $n = 16$; $P < 0.02$, Student's *t*-test) expressed as percent of the mean EPSP slope from 40 to 50 min after the induction relative to the mean EPSP slope during the control period (-10 to 0 min). Horizontal bars indicate the means. (C) Sample traces showing the temporal summation of NMDAR-mediated EPSCs elicited by the same stimulation protocol used for the LTP induction. Fifteen traces evoked by 30 -Hz stimulation at 10 -s intervals and a single EPSC trace (average of 10 traces) are superimposed. (D) (left) The level of Tyr-1472 phosphorylation is increased after electrical stimulation in the LA. Coronal sections including the LA were stained with the anti-phospho-Tyr1472 antibody. La, lateral amygdala. (right) Quantification of Tyr-1472 phosphorylation in the LA. $*P < 0.004$, Student's *t*-test, $n = 3$. The calibration bar, $500 \mu\text{m}$.

in the amygdala (stimulated slices, 1.57 ± 0.07 ; control slice, 1.00 ± 0.05 ; $P < 0.004$, Student's *t*-test) (Figure 3D). The data suggest that the lack of Tyr-1472 phosphorylation, rather than the Y1472F substitution, is responsible for the impaired amygdaloid synaptic plasticity in YF/YF mice.

Normal basic properties of synaptic transmission in YF/YF mice

The ratio of NMDAR-mediated EPSC amplitudes to α -amino-3-hydroxy-5-methylisoxazole-4-propionic-acid receptor (AMPA)-mediated EPSC amplitudes did not differ between WT/WT and YF/YF mice (WT/WT, $68.9 \pm 2.2\%$, $n = 9$; YF/YF, $69.3 \pm 2.9\%$, $n = 12$; $P > 0.9$, Student's *t*-test) (Figure 4A). Although the most straightforward explanation was that there was no change in NMDAR-mediated synaptic currents in YF/YF mice, one could argue that AMPAR-mediated synaptic responses were also decreased due to, for instance, a reduction of AMPAR surface expression at synapses. To examine this possibility, we recorded AMPAR-mediated miniature EPSCs (mEPSCs) in the whole-cell patch-clamp configuration from LA principal neurons. The averaged amplitudes of AMPAR-mediated mEPSCs were indistinguishable between WT/WT and YF/YF mice (WT/WT, 13.2 ± 0.7 pA, $n = 23$; YF/YF, 12.8 ± 0.8 pA, $n = 20$; $P > 0.8$, Student's *t*-test)

(Figure 4B). To obtain further support that the NMDAR-mediated response was unchanged, we examined the ratio of NMDAR-mediated mEPSC amplitudes to AMPAR-mediated mEPSC amplitudes (Figure 4C). No statistically significant difference was found in the averaged NMDAR-mediated mEPSC/AMPA-mediated mEPSC ratio between WT/WT and YF/YF mice (WT/WT, $18.1 \pm 2.9\%$, $n = 9$; YF/YF, $21.3 \pm 3.6\%$, $n = 7$; $P > 0.7$, Student's *t*-test). In addition, the *I*-*V* curves of WT/WT and YF/YF mice were virtually identical (Figure 4D), suggesting that Tyr-1472 phosphorylation did not affect the voltage-dependent Mg^{2+} block of NMDARs. Furthermore, the rise time ($P > 0.9$, Student's *t*-test) and decay time constant ($P > 0.7$, Student's *t*-test) of NMDAR-mediated EPSCs were not different between WT/WT (rise time, 8.40 ± 0.33 ms; decay time constant, 134.4 ± 9.7 ms; $n = 17$) and YF/YF (rise time, 8.60 ± 0.46 ms; decay time constant, 132.9 ± 9.4 ms; $n = 12$) mice, suggesting that the NMDAR subunit composition was not changed in YF/YF mice (Monyer *et al*, 1994). Together with our present results shown in Figure 3C, the data strongly suggest that basic properties of NMDARs are absolutely normal in YF/YF mice. Thus, the impaired amygdaloid LTP likely resulted from the abnormal signal transduction downstream of NMDAR activation.

Figure 4 Normal basic properties of synaptic transmission in YF/YF mice. (A) Sample traces (average of 10 traces) of evoked AMPAR-mediated (downward traces) and NMDAR-mediated (upward traces) EPSCs in WT/WT and YF/YF mice (left). Ratio of amplitudes of NMDAR-mediated EPSCs to those of AMPAR-mediated EPSCs (right) (WT/WT mice, $n = 9$; YF/YF mice, $n = 12$). The ratio was calculated for each cell, and the values were then averaged for all cells. (B) Unaltered AMPAR-mediated mEPSCs in YF/YF mice. Sample traces showing multiple events on a slower time scale (top). Averaged traces of mEPSCs recorded from single neurons (consecutive 100 events are averaged for WT/WT mice; consecutive 82 events for YF/YF mice) (bottom, left). Summary of the mean amplitudes of AMPAR-mediated mEPSCs (bottom, right) in WT/WT ($n = 23$) and YF/YF mice ($n = 20$). Horizontal bars indicate the means. (C) Sample traces of AMPAR-mediated mEPSCs (faster traces) and AMPAR-plus NMDAR-mediated mEPSCs (slower traces) in WT/WT (average of 28 traces for AMPA and NMDA mEPSCs; average of 50 traces for AMPA mEPSCs) and YF/YF (average of 53 traces for both AMPA and NMDA mEPSCs and AMPA mEPSCs) mice (left). Ratio of the mean amplitude of NMDAR-mediated mEPSCs (AMPA mEPSCs were digitally subtracted from AMPA plus NMDA mEPSCs) to that of AMPAR-mediated mEPSCs (WT/WT, $n = 9$; YF/YF, $n = 7$). Horizontal bars indicate the means. (D) Current-voltage relationships of evoked NMDA synaptic currents recorded in the presence of $10 \mu\text{M}$ CNQX in WT/WT ($n = 5$) and YF/YF ($n = 5$) mice. The current values were normalized to the value at $+34$ mV in each cell, and the values were then averaged for all cells. The liquid junction potential was compensated.

Figure 5 Impaired NMDAR-mediated CaMKII signaling in YF/YF mice. (A) Interaction of CaMKII with the WT NR2B, but very little interaction with Y1472FNR2B. NR2B immunoprecipitates (top) and lysates (bottom) were probed with the anti-CaMKII mAb. (B) Impaired glutamate-induced upregulation of CaMKII in YF/YF mice. Stimulation of coronal sections including the LA with glutamate was performed as described in Materials and methods. (C) Quantification of phospho-CaMKII in WT/WT ($n=6$ from 3 mice) and YF/YF mice ($n=6$ from 3 mice). * $P<0.01$, Student's *t*-test.

Impaired NMDAR-mediated CaMKII signaling in YF/YF mice

We then examined the NMDAR-mediated downstream signaling in WT/WT and YF/YF mice. We immunoprecipitated NMDARs from the amygdala with an antibody against the NR2B subunit and probed for the levels of co-immunoprecipitated NMDAR-associated proteins. Among these molecules, we found that CaMKII, an essential molecule for the induction of NMDAR-dependent LTP in the amygdala (Bayer *et al*, 2001; Lisman *et al*, 2002; Rodrigues *et al*, 2004), was almost absent from the NMDAR complex in YF/YF mice (Figure 5A). Consistently, we found that glutamate-induced upregulation of CaMKII was severely impaired in YF/YF mice (WT/WT, $180 \pm 20\%$, $n=3$ from 3 mice; YF/YF, $115 \pm 7\%$, $n=3$ from 3 mice; $P<0.01$, Student's *t*-test) (Figure 5B and C). We found no differences between WT/WT and YF/YF mice in glutamate-induced upregulation of ERK and CREB (Figure 5B). Other NR2B-interacting signaling molecules such as PLC γ 1 and RasGRF1 (Krapivinsky *et al*, 2003) interacted well with both the WT NR2B subunit and Y1472FNR2B (data not shown). These results suggest that the impaired LTP in YF/YF mice is caused by the impaired NMDAR-mediated CaMKII signaling in YF/YF mice.

Improper localization of the NMDAR at synaptic and perisynaptic regions in YF/YF mice

Finally, we examined the structure of synapses and the distribution of NMDAR at synapses in detail by electron microscopy. While we found no obvious abnormalities in synaptic structure of the LA in YF/YF mice (data not shown), we found the improper localization of the NR2B and NR1 subunits at asymmetrical axo-spinous synapses in the LA by postembedding immunogold staining described below (Figures 6 and 7; Supplementary Figure 4). When analyzing cross-sections of LA synapses by transmission electron microscopy, we divided each synapse into central, peripheral, and perisynaptic regions and scored each region for immunogold particles. While the method did not quantify the absolute two-dimensional distribution of particles across the synaptic membrane surface, it provided one-dimensional values useful for comparison. In YF/YF mice, the distribution of NR2B was skewed toward the peripheral and perisynaptic regions, whereas, in WT/WT mice, NR2B was located more centrally at synapses, as reported previously (Racca *et al*, 2000; Figure 6A). The distribution ratios of central to peripheral NR2B signals were 1.85 ± 0.22 for WT/WT mice and 0.99 ± 0.25 for YF/YF mice ($P<0.01$, Student's *t*-test) (Figure 6B and C). The perisynaptic region, where NMDARs undergo endocytosis (Blanpied *et al*, 2002; Petralia *et al*, 2003; Racz *et al*, 2004), was similarly enriched for NR2B in YF/YF mice (Figure 6A). The proportions of the total signal in this region were $4.36 \pm 1.42\%$ in WT/WT mice versus $11.72 \pm 4.19\%$ in YF/YF mice ($P<0.03$, Student's *t*-test) (Figure 6B and D). As for NR2B signals detected below the synaptic membrane, their perpendicular distribution from the membrane did not differ between WT/WT and YF/YF mice (Figure 6E). Furthermore, WT/WT and YF/YF mice were indistinguishable with regard to the total number of NR2B signals present per profile of asymmetrical axo-spinous synapses (Figure 6F). The distribution of NR1 was also skewed toward the peripheral and perisynaptic regions in YF/YF mice (Figure 7A–D; Supplementary Figure 4). In contrast to NR2B and NR1 subunits, the distributions and total number of NR2A signals were unchanged in YF/YF mice (Figure 7E–H; Supplementary Figure 6). These data suggest that the Y1472F mutation interferes with proper localization of NMDARs across synaptic and perisynaptic regions.

Discussion

We find that the mice carrying the mutation on the NR2B tyrosine phosphorylation site (YF/YF mice) show impaired fear learning and amygdaloid LTP. We also demonstrate that the YF/YF mice show improper localization of NMDARs at synapses. Our present findings strongly argue that proper localization of NMDARs at synapses depends on Tyr-1472 phosphorylation of NR2B, and that the proper localization may be essential for synaptic plasticity and fear learning.

We provide *in vivo* evidence that Tyr-1472 phosphorylation site is required for fear learning (Figure 2). YF/YF mice showed impaired fear learning 1 h after conditioning, suggesting that Tyr-1472 phosphorylation is essential to acquire the CS–US association during auditory fear conditioning. This observation is consistent with the report that intra-amygdala blockade of NR2B disrupts the acquisition of fear conditioning (Rodrigues *et al*, 2001). YF/YF mice have normal sensi-

Figure 6 Improper localization of the NR2B subunit in synaptic and perisynaptic regions in YF/YF mice. **(A)** Postembedding immunogold staining of NR2B subunits at asymmetrical axo-spinous synapses in the LA of WT/WT (WT/WT1 in B) and YF/YF (YF/YF1 in B) mice. NT, nerve terminal; Sp, spine. The calibration bar, 50 nm. **(B–D)** Tangential distribution of the NR2B subunit (WT/WT, 88 synapses; YF/YF, 102 synapses). **(B)** Histograms for the tangential distribution of NR2B subunits at synapses of WT/WT and YF/YF mice. Arrows indicate a boundary of the PSD. **(C)** Ratio of the number of immunogold particles for the NR2B subunit in the central region to that in the peripheral region. * $P < 0.01$, Student's *t*-test. **(D)** The number of immunogold particles for the NR2B subunit in the perisynaptic regions. Results are expressed as percent of the total number of immunogold particles at synapses. * $P < 0.03$, Student's *t*-test. **(E)** Histograms for the perpendicular distribution of NR2B subunits at synapses of WT/WT (66 synapses) and YF/YF mice (72 synapses). **(F)** The number of immunogold particles for the NR2B subunit per profile of synapses in the LA of the WT/WT (447 synapses) and YF/YF (451 synapses) mice. $P > 0.7$, Student's *t*-test.

tivity to footshock and normal context-dependent freezing (data not shown), suggesting that the impaired fear-learning in YF/YF mice cannot be explained by impaired pain perception or impaired expression of freezing behavior. YF/YF mice normally respond to the tone in an acoustic startle response test (Supplementary Figure 2), suggesting that YF/YF mice have normal auditory sense. Furthermore, YF/YF mice show impaired visual cue/footshock association (Supplementary Figure 1), confirming that YF/YF mice show impaired fear

learning. We find that the lack of Tyr-1472 phosphorylation leads to impaired LTP of the thalamo-amygdala pathway that transmits CS information to the LA (Figure 3). It is well accepted that the amygdaloid NMDAR is critical for the expression of conditioned fear responses and that the LTP of the thalamo-amygdala pathway is essential for auditory fear conditioning in mice (Gewirtz and Davis, 1997; Lee and Kim, 1998; for a review, see LeDoux, 2000; Fendt, 2001; Rodrigues *et al*, 2001; Bauer *et al*, 2002). Thus, we speculate

Figure 7 Improper localization of NR1 but normal localization of NR2A in synaptic and perisynaptic regions in YF/YF mice. (A) Postembedding immunogold staining of the NR1 subunit at asymmetrical axo-spinous synapses in the LA of WT/WT (WT/WT1 in Supplementary Figure 4A) and YF/YF (YF/YF1 in Supplementary Figure 4A) mice. NT, nerve terminal; Sp, spine. The calibration bar, 50 nm. (B, C) Tangential distribution of the NR1 subunit (WT/WT, 81 synapses; YF/YF, 84 synapses). (B) Ratio of the number of immunogold particles for the NR1 subunit in the central region to that in the peripheral region (WT/WT, 2.70 ± 0.88 ; YF/YF, 0.79 ± 0.14 ; $*P < 0.02$, Student's *t*-test). (C) The number of immunogold particles for the NR1 subunit in the perisynaptic regions. Results are expressed as percent of the total number of immunogold particles at synapses (WT/WT, $2.27 \pm 1.63\%$; YF/YF, $8.47 \pm 3.89\%$; $*P < 0.03$, Student's *t*-test). (D) The number of immunogold particles for the NR1 subunit per profile of synapses in the LA of WT/WT (2.45 ± 0.43 , 100 synapses) and YF/YF (2.07 ± 0.20 , 100 synapses; $P > 0.2$, Student's *t*-test) mice. (E) Postembedding immunogold staining of the NR2A subunit at asymmetrical axo-spinous synapses in the LA of WT/WT (WT/WT1 in Supplementary Figure 4C) and YF/YF (YF/YF1 in Supplementary Figure 4C) mice. The calibration bar, 50 nm. (F, G) Tangential distribution of the NR2A subunit (WT/WT, 70 synapses; YF/YF, 55 synapses). (F) Ratio of the number of immunogold particles for the NR2A subunit in the central region to that in the peripheral region (WT/WT, 1.69 ± 0.36 ; YF/YF, 1.59 ± 0.25 ; $P > 0.7$, Student's *t*-test). (G) The number of immunogold particles for the NR2A subunit in the perisynaptic regions (WT/WT, $5.38 \pm 3.15\%$; YF/YF, $3.55 \pm 1.51\%$; $P > 0.2$, Student's *t*-test). (H) The number of immunogold particles for the NR2A subunit per profile of synapses in the LA of WT/WT (2.32 ± 0.15 , 70 synapses) and YF/YF (2.35 ± 0.16 , 55 synapses; $P > 0.7$, Student's *t*-test) mice.

amygdala is involved in the impaired fear learning of YF/YF mice but further study is needed to prove this.

Our present findings strongly suggest that basic properties of NMDAR channels are absolutely normal in YF/YF mice, indicating that the impaired LTP in YF/YF mice is due to the deficits in the signal downstream from NMDAR activation. Given that the components of the NMDAR complex show spatially restricted localization in the PSD (Kornau *et al*, 1997; Husi *et al*, 2000; Scannevin and Huganir, 2000; Sheng and Kim, 2002), it is possible that the improperly localized Y1472FNR2B in the peripheral and perisynaptic regions cannot organize the proper NMDAR signaling complex. We have found that NMDAR-associated CaMKII is dramatically reduced and that glutamate-induced upregulation of CaMKII was severely impaired in YF/YF mice (Figure 5). Because CaMKII is an essential protein for the induction of NMDAR-dependent LTP in the amygdala and the interaction between NR2B and CaMKII is required for synaptic plasticity (Lisman *et al*, 2002; Rodrigues *et al*, 2004; Barria and Malinow, 2005), the impaired LTP in YF/YF mice is likely to be caused by the impaired NMDAR-mediated CaMKII signaling in YF/YF mice.

We did not detect any major abnormalities in hippocampal synaptic plasticity and hippocampus-dependent learning using several standard protocols (data not shown). It is possible that NR2A, one of the members of NR2 family, has overlapping functions in the hippocampus, but not in the amygdala. Indeed, NR2A knockout mice show impairments in a hippocampus-dependent contextual fear-conditioning task, while these mice are normal in the performance of amygdala-dependent auditory fear conditioning (Kiyama *et al*, 1998). Alternatively, in the hippocampus, some compensatory event might have occurred to cover the hippocampal phenotypes in the YF/YF mice. Under specific conditions, we may detect abnormalities in hippocampal functions in YF/YF mice. In addition, Tyr-1472 phosphorylation may regulate synaptic functions in other brain areas such as nucleus accumbens and striatum.

Our data clearly demonstrate that localization of the NR1 subunit, an essential subunit for NMDARs, is determined by the NR2B subunit (Figure 7). In contrast to NR2B, the distribution and total number of NR2A at synapses is unaltered in YF/YF mice. These results indicate that the NR2B subunit is present at mature synapses and required for proper allocation of the heteromeric NMDAR complex at central synapses *in vivo*, although it has been reported that the NR2B protein is gradually replaced by the NR2A protein during LA development (Lopez de Armentia and Sah, 2003). In the adult amygdala, the NR1/NR2B complex, but not the NR1/NR2A or NR1/NR2A/NR2B complex, may be the major NMDAR complex and contribute to amygdaloid functions more closely than the other complex, and thus it is possible that Tyr-1472 phosphorylation of the NR2B subunit exerts more influence on synaptic plasticity in the amygdala than in the hippocampus.

We provide *in vivo* evidence suggesting that the Tyr-1472 phosphorylation site is relevant to proper localization of NMDARs at synapses and to regulation of the interaction of NMDARs with α -actinin2 and the AP-2 complex (Supplementary Figure 5). In WT/WT mice, NMDARs are located centrally at synapses (Figures 6 and 7) and a small portion of NR2B moves laterally at synapses in the basal state (Racca *et al*, 2000; Tovar and Westbrook, 2002). Our present

results suggest that the Tyr-1472-phosphorylated NR2B is tightly associated with α -actinin2 to anchor NMDARs to the subsynaptic actin cytoskeleton. Such a link would be necessary for the central localization of NMDARs at synapses. On dephosphorylation at Tyr-1472, NR2B subunits would no longer be associated with postsynaptic actin filaments, allowing NMDARs to move laterally at synapses. Given that the surface NMDARs undergo endocytosis at the endocytotic zone (perisynaptic region) by interacting with the AP-2 complex in response to external stimuli (Roche *et al*, 2001; Vissel *et al*, 2001; Blanpied *et al*, 2002; Petralia *et al*, 2003; Racz *et al*, 2004), some portion of Tyr-1472-dephosphorylated NR2B can enter the perisynaptic region to be endocytosed. Replacing endocytosed NMDARs, newly assembled NMDARs are delivered to the synapses (Carroll and Zukin, 2002). In contrast, Y1472FNR2B in YF/YF mice interacts inefficiently with both α -actinin2 and the AP-2 complex (Supplementary Figure 5), leading to the scattered localization of Y1472FNR2B at synapses and to accumulation of Y1472FNR2B in the perisynaptic regions where WT NR2B would normally be endocytosed (Blanpied *et al*, 2002; Petralia *et al*, 2003; Racz *et al*, 2004; Figure 6). Although it remains unclear how Tyr-1472 phosphorylation regulates these interactions, Tyr-1472 phosphorylation has at least two modes of binding, one in which phosphorylation of Tyr-1472 allows binding to α -actinin2, and the other in which dephosphorylation of Tyr-1472 allows binding to the AP-2 complex. Because the AP-2 complex interacts directly with its substrate via tyrosine residues (Ohno *et al*, 1995), Tyr-1472 phosphorylation would negatively regulate direct interaction of the NR2B subunit with the AP-2 complex (Lavezzari *et al*, 2003). α -Actinin2 may also interact with Tyr-1472-phosphorylated NR2B directly. Alternatively, some scaffolding protein may exist to link NR2B and α -actinin2.

A recent study suggests that extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways (Hardingham *et al*, 2002; Vanhoutte and Bading, 2003). Although Y1472F NR2B-containing NMDARs show improper localization at synapses (Figures 6 and 7), they are still associated with PSD as shown in the EM analysis. Furthermore, glutamate-induced CREB phosphorylation and cell death were normal in YF/YF mice (Figure 5; Supplementary Figure 7). Thus, these results strongly suggest that most Y1472FNR2B-containing NMDARs are in the synaptic and perisynaptic region, but not at the extrasynaptic region.

While a considerable number of NMDARs are in the perisynaptic regions in YF/YF mice (Figures 6 and 7), we observe no change in the NMDAR-mediated responses (Figure 4). Given that the NMDAR has a higher affinity for glutamate (Kullmann and Asztely, 1998), despite the relatively long distance from the presynaptic terminals to the perisynaptic Y1472FNR2B, improperly localized Y1472FNR2B may be able to sense almost the same amount of glutamate as WT NR2B. Thus, the basic properties of NMDAR may be unaltered in YF/YF mice.

Previously, we have found that the level of Tyr-1472 phosphorylation increases in parallel with brain development (Nakazawa *et al*, 2001). Recent reports have also shown that the level of tyrosine phosphorylation of the NR2B subunit increases after novel taste learning or the induction of hippocampal LTP (Salter and Kalia, 2004). Therefore,

Tyr-1472 phosphorylation plays significant roles at relatively mature synapses. Indeed, YF/YF mice appear healthy in general behaviors, whereas mice lacking NR2B subunits or those expressing the C-terminal-truncated NR2B subunit die soon after their birth, suggesting that the tyrosine phosphorylation itself is not essential for early development (Mori *et al*, 1998; Sprengel *et al*, 1998). In addition, Tyr-1472 phosphorylation is implicated not only in learning and memory but also in ischemia and ethanol sensitivity (Salter and Kalia, 2004). Therefore, the knockin mice will also be useful for the analysis of the role of NMDARs on other diverse brain functions.

Recently, we have reported that the level of Tyr-1472 phosphorylation in *fyn*-deficient mice is much less than that in WT mice (Nakazawa *et al*, 2001). Consistent with our present data, *fyn*-deficient mice show impaired amygdala-dependent behaviors (Miyakawa *et al*, 1994). Interestingly, the transgenic mice, in which *Fyn* is overexpressed and the level of tyrosine phosphorylation of the NR2B subunit is elevated, also show impaired auditory fear learning (Kojima *et al*, 2005). Thus, proper regulation of Tyr-1472 phosphorylation may be critical for amygdala-dependent fear learning. We propose that the activity-dependent phosphorylation of the Tyr-1472 is a molecular switch that regulates fear-related memory formation.

Materials and methods

Generation of YF/YF mice

See Supplementary data for methods describing the generation YF/YF mice.

Antibodies

Polyclonal antibodies against phospho-Tyr-1472, NR1, NR2B, NR2A and PSD-95 were described previously (Watanabe *et al*, 1998; Nakazawa *et al*, 2001; Fukaya *et al*, 2003). The commercially available antibodies used in this study are described in Supplementary data.

Preparation of lysates, immunoprecipitation and immunoblotting

Preparation of lysates from amygdaloid slices and whole telencephalons, immunoprecipitation and immunoblotting were performed as described previously (Nakazawa *et al*, 2001, 2003). For quantification, the immunoreacted protein bands were analyzed with the NIH image software.

Auditory fear conditioning

Fear conditioning was conducted in a small conditioning chamber surrounded by a sound-attenuating chest (CL-M3, O'Hara & Co., Ltd, Tokyo, Japan). Mice were placed in the conditioning chamber for 170 s and then presented with a tone of 65 dB/10 kHz for 10 s through a speaker on the ceiling of the sound-attenuating chamber. At the end of the tone presentation, the mice were given a footshock (2 s/0.35 mA). Freezing responses were monitored for 1 min after the footshock, and the mice were then returned to their home cages. On the test day, the mice were placed in a novel chamber with contexts different from those of the conditioning chamber to minimize freezing caused by contextual fear conditioning. Freezing was scored for 3 min before delivery of the tone and then for 3 min in the presence of the tone. Freezing responses were analyzed on a Macintosh computer with Image FZC 2.22sr2 (O'Hara & Co., Ltd), the software based on the NIH Image program.

Electrophysiology

Whole-cell recordings were performed as described previously (Manabe *et al*, 2000). Coronal slices including the LA (400- μ m thickness) were prepared from 4- to 6-week-old mice and placed in an interface-type holding chamber for at least 1 h. A slice was then

transferred to the recording chamber and submerged beneath continuously perfusing artificial cerebrospinal fluid (ACSF) consisting of (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 2.5 CaCl₂, 1.0 NaH₂PO₄, 26.2 NaH₂CO₃, 11 glucose, which had been saturated with 95% O₂ and 5% CO₂. All the perfusing solutions contained 100 µM picrotoxin (Wako Pure Chemical Industries, Osaka, Japan) to block GABA_A receptor-mediated inhibitory synaptic responses. The dorsal region of the cortex was cut off to prevent the invasion of epileptiform activity. A whole-cell pipette was placed in the LA. For current-clamp recordings, the whole-cell pipette was filled with the internal solution containing (in mM): 135 potassium methanesulfonate, 8 NaCl, 10 HEPES, 2 Mg-ATP, and 0.3 Na₃-GTP (pH 7.3; 290–300 mOsm). For voltage-clamp recordings, the internal solution contained (in mM): 122.5 cesium gluconate, 17.5 CsCl, 10 HEPES, 0.2 EGTA, 8 NaCl, 2 Mg-ATP and 0.3 Na₃-GTP (pH 7.2; 290–300 mOsm). For current-clamp experiments, cells were clamped at –75 mV with DC current injection. To stimulate the presynaptic thalamic fibers, a tungsten bipolar electrode was placed in the ventral striatum just medial to the LA. The test stimuli were applied to the thalamic pathway at 0.1 Hz, and the stimulus strength was adjusted to evoke excitatory postsynaptic potentials (EPSPs) with amplitudes of 3–5 mV. To induce LTP, trains of 10 stimuli at 30 Hz were paired with depolarization (1 nA/8 ms) given 5 ms after the onset of each EPSP in the train. This pairing was given 15 times at 10-s intervals. For voltage-clamp experiments, AMPA receptor-mediated EPSCs were recorded at –80 mV, and NMDA receptor-mediated EPSCs were recorded at +40 mV with the same stimulus strength in the presence of 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris, Avonmouth, UK). MEPSs were recorded in the presence of 1 µM tetrodotoxin (Sankyo, Tokyo, Japan) to block action potentials. Cells were clamped at –80 mV in the presence of Mg²⁺ (1.3 mM) to measure the AMPAR-mediated component, and then the external solution was switched to the nominally Mg²⁺-free solution. After washing out external Mg²⁺ (typically around 30 min), mEPSCs composed of both D-APV-sensitive and -insensitive components were observed. D-APV (50 µM; Tocris, Avonmouth, UK) was then applied to block the NMDAR-mediated component. MEPSs were analyzed using the Mini Analysis (6.0.3) software. For recording AMPAR-mediated mEPSCs, the detection threshold was set at 10 pA, while it was increased to 21–25 pA for recording simultaneously both AMPAR-mediated and NMDAR-mediated mEPSCs partly because the background noise level was increased in the absence of Mg²⁺. Axoclamp 2B, Axopatch 1D or MultiClamp 700A amplifier, and Digidata 1320A or Digidata 1322A (Axon Instruments, Union City, CA) were used to record and store the data with the pClamp software (Axon Instruments, Union City, CA), respectively. The signal was filtered at 2–6 kHz and digitized at

10 kHz. All experiments were performed at 25–28°C. All data are presented as the mean ± s.e.m.

Histology

Immunohistochemical analyses were performed as described previously (Fukaya *et al*, 2003).

Pharmacological treatment of brain slices

Coronal slices including the LA (400-µm thickness) were prepared from 4- to 6-week-old mice and placed in an interface-type holding chamber for at least 3 h. Slices were preincubated in ACSF with 1 µM TTX (Wako) and 1 µM okadaic acid (Calbiochem, San Diego, CA) for 1 h and then stimulated with 10 µM glutamate and 10 µM glycine for 7 min. Preparations of cell lysates were described above.

Immunogold electron microscopy

Postembedding immunogold analysis was performed as described previously (Fukaya *et al*, 2003). To carry out a semiquantitative distribution analysis of the NR2A, NR2B or NR1 subunit across synapses, synapse cross-section lengths were measured, and any shorter than 150 nm were excluded to avoid cross-sections lying too far off-center. Immunogold particles at remaining axo-synaptic asymmetrical synapses in the LA were counted by taking the central 50% of the total PSD cross-section length as the 'central' region, and the two ends, each 25% of the total PSD cross-section length, as the 'peripheral' region. An additional 20% length outside the PSD on each side was defined as the 'perisynaptic' region.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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