Supplementary data

Involvement of NMDAR2A tyrosine phosphorylation in depression-related behavior

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Results

Characterization of the rabbit polyclonal antibody against Tyr-1325 phosphorylated-NR2A subunits

Antisera that recognize Tyr-1325 phosphorylated-NR2A subunits were raised by immunizing rabbits with the phosphotyrosine-containing synthetic peptide corresponding to the amino acid sequence of the NR2A subunit surrounding Tyr-1325. Antisera were extensively preabsorbed with non-phosphorylated GST-C2 protein that contained Tyr-1325 and then affinity-purified with the synthetic phosphopeptide used as immunogen. We first examined whether the purified antibody recognized Tyr-1325 phosphorylated-NR2A subunits using the recombinant NR2A subunit expressed in HEK293T cells. HEK293T cells were transfected with expression plasmids encoding active Fyn and wild-type or Y1325F mutant of NR2A subunits. Western blots of NR2A immunoprecipitates from the wild-type or Y1325F NR2A expressing cells with the

anti-phospho-Tyr1325 antibody showed their specific reactivity with tyrosine phosphorylated wild-type-NR2A subunit but not with tyrosine-phosphorylated Y1325F-NR2A or non-phosphorylated-NR2A subunits (Supplementary Figure 1A). Thus, the anti-phospho-Tyr-1325 antibody raised in this study was likely to specifically recognize Tyr-1325 phosphorylated-NR2A subunits. To further examine the specificity of the antibody, we used extracts of the mouse telencephalon. The NR2A subunit was immunoprecipitated from the extracts and subjected to immunoblotting with the anti-phospho-Tyr-1325 antibody. The antibody reacted with a protein corresponding to Tyr-1325 phosphorylated-NR2A subunits (Supplementary Figure 1B). Significantly, this immunoreactivity was completely abolished when the immunoprecipitated NR2A subunit was treated with bacterial alkaline phosphatase (Supplementary Figure 1B). Therefore, we concluded that the anti-Tyr-1325 antibody raised in this study specifically recognized Tyr-1325 phosphorylated-NR2A subunits.

Normal general behaviors such as cognitive function, startle response, and pain behavior in YF/YF mice

We performed a battery of behavioral tests to examine cognitive function, startle response, and pain behavior of YF/YF mice (Supplementary Figure 2-4). We did not detect any significant abnormalities of YF/YF mice in the Morris water maze test (Supplementary Figure 2A-C), contextual fear conditioning test (Supplementary Figure 2D and E), auditory fear conditioning test (Supplementary Figure 2D and F), startle response test (Supplementary Figure 3A), pre-pulse inhibition test (Supplementary Figure 3B), hot plate test (Supplementary Figure 4A), and tail flick test (Supplementary Figure 4B). These results suggest that general behaviors such as cognitive function,

startle response, and pain behavior are normal in YF/YF mice.

Little effect of the elevated plus maze test and the open field test on Tyr-1325 phosphorylation

Five minutes after the elevated plus maze test, the mice were sacrificed, the striata were dissected out, and the lysates were prepared. The NR2A immunoprecipitates from these lysates were then subjected to immunoblotting with the anti-pY1325 antibody followed by re-blot with the anti-NR2A antibody. As shown in Supplementary Figure 5A, the level of Tyr-1325 phosphorylation in the striatum was little affected upon exposure to the elevated plus maze. Likewise, Tyr-1325 phosphorylation in the striatum was unchanged by exposure to the open field test (Supplementary Figure 5B).

Normal hippocampal synaptic plasticity in acute slices from YF/YF mice

We examined the effect of Y1325F mutation on hippocampal synaptic plasticity (Supplementary Figure 6). Tetanic stimulation of the Schaffer collateral inputs into the CA1 region of the hippocampus (100 Hz, 1s) induced robust long-term potentiation (LTP) in acute slices from YF/YF mice, which was indistinguishable from that in acute slices from WT/WT mice (Supplementary Figure 6). Thus, NMDA receptor-dependent LTP induction with the standard protocol is normal in YF/YF mice.

Normal NMDA receptor-mediated signaling in the prefrontal cortex of YF/YF mice

We examined the NMDA receptor-mediated downstream signaling in the prefrontal cortex of WT/WT and YF/YF mice (Supplementary Figure 7). Prefrontal cortex culture from 17-day-old embryonic mice was prepared as described in Materials and Methods.

Then neurons were stimulated with 10 μM NMDA for 10 min at 11 DIV (days *in vitro*). We did not detect significant abnormalities of YF/YF mice in the NMDA receptor-induced up-regulation of phospho-ERK and phospho-CREB (Supplementary Figure 7), suggesting that the NMDA receptor-mediated signaling in the prefrontal cortex is normal in YF/YF mice.

Unchanged tyrosine phosphorylation of the Y1325F-containing NR2A subunit by the forced swim test

Five minutes after the forced swim test, YF/YF mice were sacrificed, the striata were dissected out, and the lysates were prepared. The Y1325F-NR2A immunoprecipitates from these lysates were then subjected to immunoblotting with the anti-phospho-tyrosine antibody (4G10) followed by reblot with the anti-NR2A antibody. As shown in Supplementary Figure 8, we could not detect significant increase in the level of tyrosine phosphorylation of the Y1325F-containing NR2A subunit by the forced swim test, suggesting that any of tyrosine phosphorylation sites except for Tyr-1325 in the NR2A subunit is insensitive to the forced swim test.

Normal PKA activity in the striatum of YF/YF mice

We measured PKA activity in the striatum as described in Materials and Methods. We could not detect significant abnormalities in PKA activity in YF/YF mice compared with that in WT/WT mice (Supplementary Figure 9).

Materials and Methods

Phosphatase treatment of NR2A immunoprecipitates

NR2A immunoprecipitates were incubated with bacterial alkaline phosphatase (10 unit) (Takara, Ohtsu, Japan) at 37 ˚C for 6 h (Nakazawa *et al*, 2001).

Morris water maze test

The visible platform and hidden platform versions of the Morris water maze test were conducted to assess spatial learning ability. The apparatus (WM-3002; O' Hara & Co., Ltd, Tokyo, Japan) consisted of a circular tank (30 cm H × 100 cm in diameter) filled with water (22-cm deep) maintained at room temperature (22-24°C) and made opaque with nontoxic white paint. The surface of the platform (10 cm in diameter) was 1 cm below the water surface. In the visible platform test, six trials per day were conducted for 2 successive days. There were four possible locations for the platform. The location of the visible platform was changed every trial. The latency to reach the platform was recorded. In the hidden platform, four trials per day were conducted for 6 successive days. One of these platform positions was assigned to each mouse as correct location during the training. The latency to reach the platform, the distance traveled to reach the platform, and average swim speed were recorded. On the 6th day of the training, the platform was removed, and a probe trial was conducted for 60 s. The time spent in each quadrant, the number of crossings above the former target site, and average swim speed were recorded during the probe trials. Data acquisition and analysis were performed using Image WM software (O'Hara & Co., Ltd).

Contextual and auditory fear conditioning tests

Fear conditioning was conducted in a small conditioning chamber surrounded by a sound-attenuating chest (CL-M3, O'Hara & Co., Ltd., Tokyo, Japan). On day 1, mice

were placed in the conditioning chamber for 10 s and then presented with a tone of 65 dB/10 kHz for 10 s through a speaker on the roof of the chest. At the end of the tone presentation, a footshock (2 s/0.35 mA) was paired with the tone. Freezing responses were monitored for 1 min after the footshock, and the mice were then returned to their home cages. On day 2, the mice were placed in the conditioning chamber and freezing was scored for 6min. On day 3, 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 presenting the tone and then for 3 min in the presence of the tone. Freezing responses were analyzed with Image FZC 2.22sr2 software (O'Hara & Co., Ltd).

Acoustic startle response and pre-pulse inhibition tests

The startle reflex measurement system (SM-1001; Kinder Scientific, San Diego, CA, USA) was used for assessing acoustic startle responses and pre-pulse inhibition. A test session began by placing a mouse in a Plexiglas chamber where it was left undisturbed for 2 min. The duration of white noise that was used as the startle stimulus was 40 ms for all trial types. The startle response was recorded for 200 ms (measuring the response every 1 ms) starting with the onset of the startle stimulus. The background noise level in the chamber was 70 dB. The peak startle amplitude during the 200 ms sampling window was recorded as a measure of the startle response. A test session consisted of seven trial types (i.e., five types for startle stimulus-only trials and two types for pre-pulse inhibition trials). The intensity of startle stimulus was 70, 90, 100, 110 and 120 dB. The pre-pulse sound was presented 100 ms before the startle stimulus, and its intensity was 75 or 80 dB. Eight blocks of the seven trial types were presented in pseudorandom order

such that each trial type was presented once within a block. The average intertrial interval was 15 s (range, 10-20 s). The following formula was used to calculate percentage pre-pulse inhibition of the startle response: 100 - [100× (startle response on pre-pulse trials/startle response on 120dB startle trials)].

Tail flick test

The animals were gently held by hand such that the tail was placed in the groove of the tail-flick unit (Model 7360, Ugo Basile, Comerio, Italy). The light was focused on the tail 1.5 cm from its tip by a foot-operated switch. The tail-flick latencies were recorded and a cutoff time was set to 15 s to prevent thermal injury. Tail flick reactions were monitored three consecutive trials at a 10-s interval.

Hot plate test

Mice were placed on the metal surface (250 mm W × 200 mm D × 10 mm H) of an electronically controlled hot plate analgesia meter (MK305B, Muromachi Kikai, Tokyo, Japan) maintained at 50 ˚C. The time when mice began to lick their hind-paws was recorded as an indicator of the pain threshold. The latency to jump from the heated surface of the hot plate was also measured, and a cutoff time was set to 180 s to prevent thermal injury.

Electrophysiology in acute hippocampal slices

Extracellular field potential recordings were performed essentially as described previously (Bongsebandhu-phubhakdi and Manabe, 2007). Hippocampal slices (400-μm thick) were prepared from 6-8-week-old mice and placed in a holding chamber for at

least 1 h. A slice was then transferred to the recording chamber and submerged beneath continuously perfusing artificial cerebrospinal fluid (in mM: 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1.0 NaH_2PO_4, 26.2 NaH_2CO_3, and 11 glucose) that had been saturated with 95 % O_2 and 5 % CO_2. All the perfusing solutions contained 100 μM picrotoxin to block $GABA_A$ receptor-mediated inhibitory synaptic responses. The CA3 region was surgically separated from the CA1 region to prevent invasion of epileptiform activity. A glass recording electrode (containing 3-M NaCl) and a tungsten bipolar stimulating electrode were placed in the striatum radiatum. The test stimulation was applied to Schaffer collateral fibers at 0.1 Hz. The stimulus strength was adjusted to evoke excitatory postsynaptic potentials (EPSPs) of the maximal initial slope value of 0.10-0.15 mV/ms. To induce LTP, we applied tetanic stimulation (100 Hz, 1 s) to Schaffer collateral fibers. An Axopatch-1D amplifier (Molecular Devices, Sunnyvale, CA, USA) was used to record synaptic responses. Data were digitized at 10 kHz and analyzed using pClamp software (Molecular Devices). All experiments were done at 25 ˚C.

Antibodies

The commercially available antibodies are anti-phosphotyrosine (4G10) (Upstate Biotechnology), anti-phospho ERK (Cell Signaling Technology, Beverly, MA, USA), anti-ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-CREB (Cell Signaling Technology), and anti-CREB (Cell Signaling Technology) antibodies.

Measurement of PKA activity

PKA activity was measured with a PKA kinase activity assay kit (Assay designs, Ann

Arbor, MI, USA).

Cell culture

Prefrontal cortex cultures from 17-day-old embryonic mice were prepared essentially as described previously (Nakazawa *et al*, 2006).

Pharmacological treatment of neurons

Prefrontal cortex neurons were stimulated with 10 μM NMDA for 10 min at 11 DIV and then cells were lysed with TNE buffer (Nakazawa *et al*, 2001).

References

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Nakazawa T, Komai S, Watabe AM, Kiyama Y, Fukaya M, Arima-Yoshida F, Horai R, Sudo K, Ebine K, Delawary M, Goto J, Umemori H, Tezuka T, Iwakura Y, Watanabe M, Yamamoto T, Manabe T (2006) NR2B tyrosine phosphorylation modulates fear learning as well as amygdaloid synaptic plasticity. *EMBO J* 25: 2867-2877

Figure Legends

Supplementary Figure 1. Characterization of the rabbit anti-phospho-Tyr-1325 polyclonal antibody used in this study. (**A**) Detection of Tyr-1325-phosphorylated NR2A subunits in HEK 293T cells using the anti-phospho-Tyr-1325 antibody. HEK 293T cells were transfected with combinations of expression plasmids encoding the NR2A subunit, various NR2A YF mutants, and active Fyn (FynYF) or inactive Fyn (Fyn KM). NR2A-immunoprecipitates from cell lysates were subjected to immunoblotting with anti-phospho-Tyr-1325 antibody, followed by reblot with the anti-NR2A antibody. The anti-phospho-Tyr-1325 antibody reacted specifically with tyrosine-phosphorylated WT-NR2A but not with tyrosine-phosphorylated Y1325F-NR2A or non-phosphorylated NR2A subunits, indicating that the anti-phospho-Tyr-1325 antibody specifically recognized Tyr-1325-phosphorylated NR2A subunits. (**B**) Detection of Tyr-1325-phosphorylated NR2A subunits in murine brain. The telencephalons from wild-type mice were homogenized in TNE buffer. NR2A immunoprecipitates from the lysates were treated with (lane 2) or without (lane 1) bacterial alkaline phosphatase (BAP) and were subjected to immunoblotting with the anti-phospho-Tyr-1325 antibody, followed by reblot with the anti-NR2A antibody.

Supplementary Figure 2. Normal hippocampus- and amygdala-dependent learning in YF/YF mice**.** (**A-C**) The Morris water maze test. (**A**) The mean speed during the probe trials. (**B**) The distance traveled during the probe trials. (**C**) The time spent in each quadrant during the probe trials. (**D-F**) The auditory and contextual fear conditioning test. (**D**) Freezing responses on the conditioning day. A tone was presented for 10 s (*solid line*) 10 s after the placement of mice in the conditioning chamber; at the end of the tone, mice were given a foot shock for 2 s (*arrow*). (**E**) Freezing responses in the

contextual fear conditioning. (**F**) Freezing responses in the auditory fear conditioning test. The same tone was presented for 3 min (*solid line*) 3 min after the placement of mice into a testing chamber with novel contexts. There were no significant differences between WT/WT and YF/YF mice in any of the parameters (WT/WT, $n = 13$; YF/YF, $n = 11$, $p > 0.05$, one-way ANOVA). n.s.: not significant.

Supplementary Figure 3. Normal acoustic startle responses and pre-pulse inhibition in YF/YF mice. (**A**) Normal responses to the acoustic stimuli in the acoustic startle response test in YF/YF mice. Mean amplitudes of startle responses are shown. (**B**) Normal pre-pulse inhibition in YF/YF mice. There were no significant differences between WT/WT and YF/YF mice in any of the parameters (WT/WT, $n = 13$; YF/YF, $n = 11$, $p > 0.05$, one-way ANOVA). n.s.: not significant.

Supplementary Figure 4. Normal pain behavior in YF/YF mice. (**A**) Normal hind-paw licking and jumping latencies in the hot plate test in YF/YF mice. (**B**) Normal tail flick latency in YF/YF mice. There were no significant differences between WT/WT and YF/YF mice in any of the parameters (WT/WT, $n = 13$; YF/YF, $n = 11$, $p > 0.05$, one-way ANOVA). n.s.: not significant.

Supplementary Figure 5. Little effect of the performance of the elevated plus maze test (EPM) and the open field test (OF) on Tyr-1325 phosphorylation. Five minutes after (**A**) the elevated plus maze test or (**B**) the open field test, the striatum was dissected out and the lysates were prepared. The NR2A immunoprecipitates from these lysates were then subjected to immunoblotting with the anti-pY1325 antibody followed by reblot

with the anti-NR2A antibody. Results showed that the level of Tyr-1325 phosphorylation in the striatum was little affected upon exposure to the elevated plus maze or the open field test. $p > 0.05$, $n = 3$; Student's t test. n.s.: not significant.

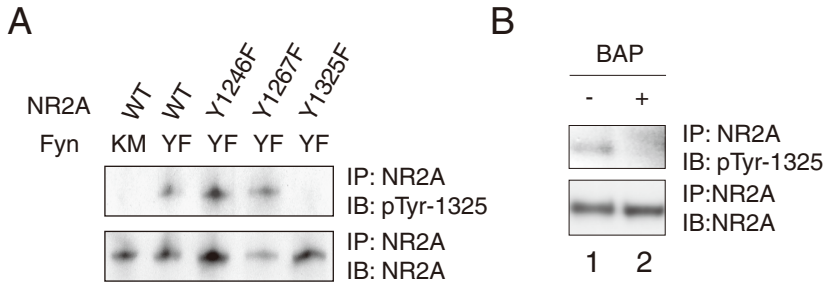
Supplementary Figure 6. Normal tetanus-induced LTP in YF/YF mice. The time course of LTP induced by single tetanic stimulation (100 Hz, 1 s). Representative traces (averaged 10 consecutive responses) in the inset are EPSPs obtained at the times indicated by the numbers in the graph (○: WT/WT, $n = 16$; ●: YF/YF, $n = 21$). Note that LTP in YF/YF mice was indistinguishable from that in WT/WT mice.

Supplementary Figure 7. Normal NMDA receptor-mediated signaling in the prefrontal cortex of YF/YF mice. (**A**) Normal NMDA receptor-mediated ERK activation in YF/YF mice. Prefrontal cortex neurons from WT/WT and YF/YF mice were stimulated with 10 μM NMDA for 10 min and homogenized in TNE buffer. Then the lysates were subjected to immunoblotting with the anti-pERK antibody, followed by reblot with the anti-ERK antibody. A representative blot is shown on the *left*. $p > 0.05$, $n = 3$, Student's t test. (**B**) Normal NMDA receptor-mediated CREB phosphorylation in YF/YF mice. The lysates of prefrontal cortex neuron were prepared as above. The lysates were subjected to immunoblotting with the anti-pCREB antibody, followed by reblot with the anti-CREB antibody. A representative blot is shown on the *left*. $p > 0.05$, $n = 3$, Student's t test. n.s.: not significant.

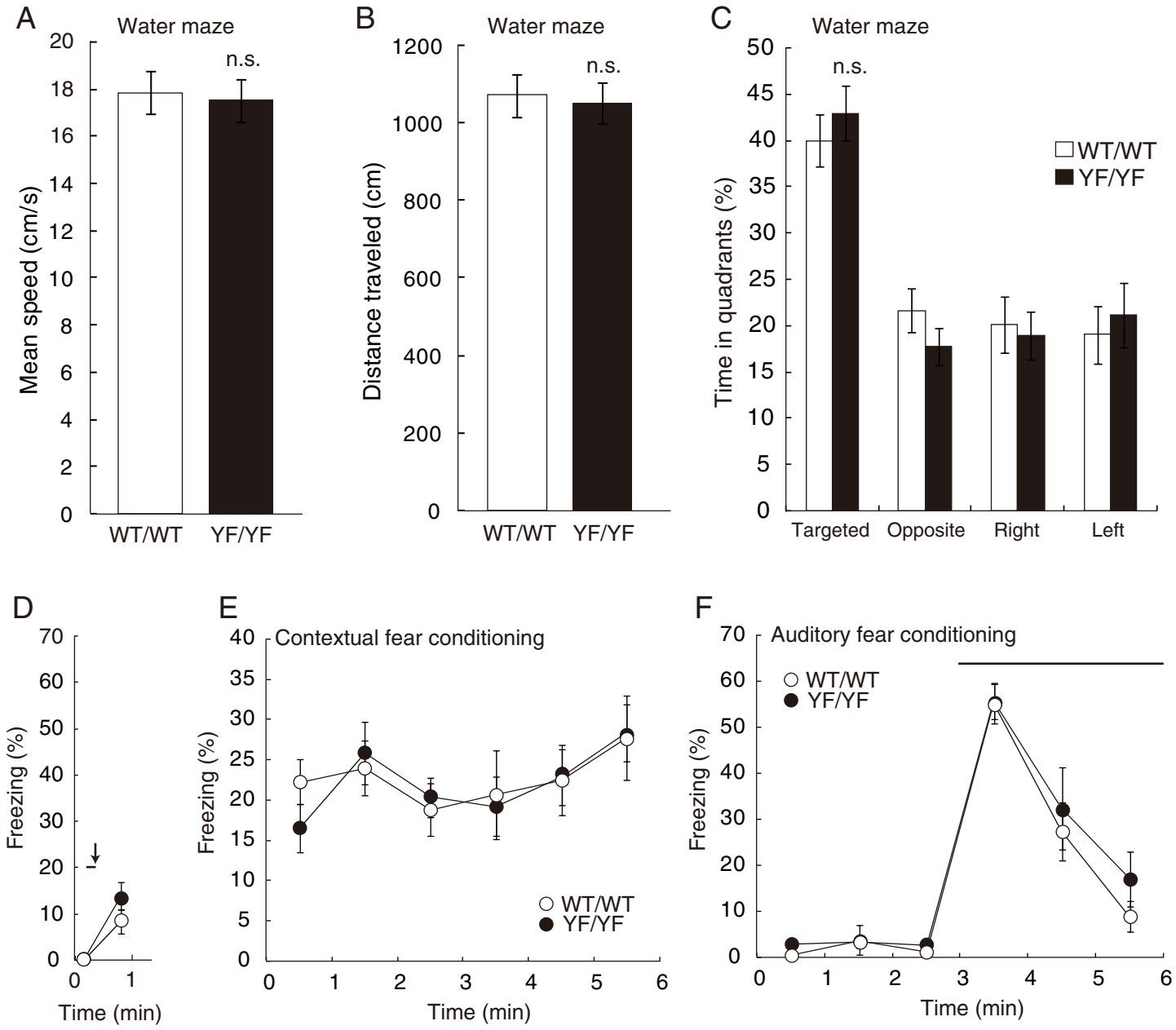
Supplementary Figure 8. Unchanged tyrosine phosphorylation of Y1325F-mutated NR2A during the forced swim test. Equal amounts of Y1325F-NR2A

immunoprecipitates from lysates of the forced swim test-exposed- and control-YF/YF mice were probed with the anti-phospho-tyrosine antibody (4G10) followed by reblot with the anti-NR2A antibody. A representative blot is shown on the *left*. $p > 0.05$, $n = 3$, Student's *t* test. n.s.: not significant.

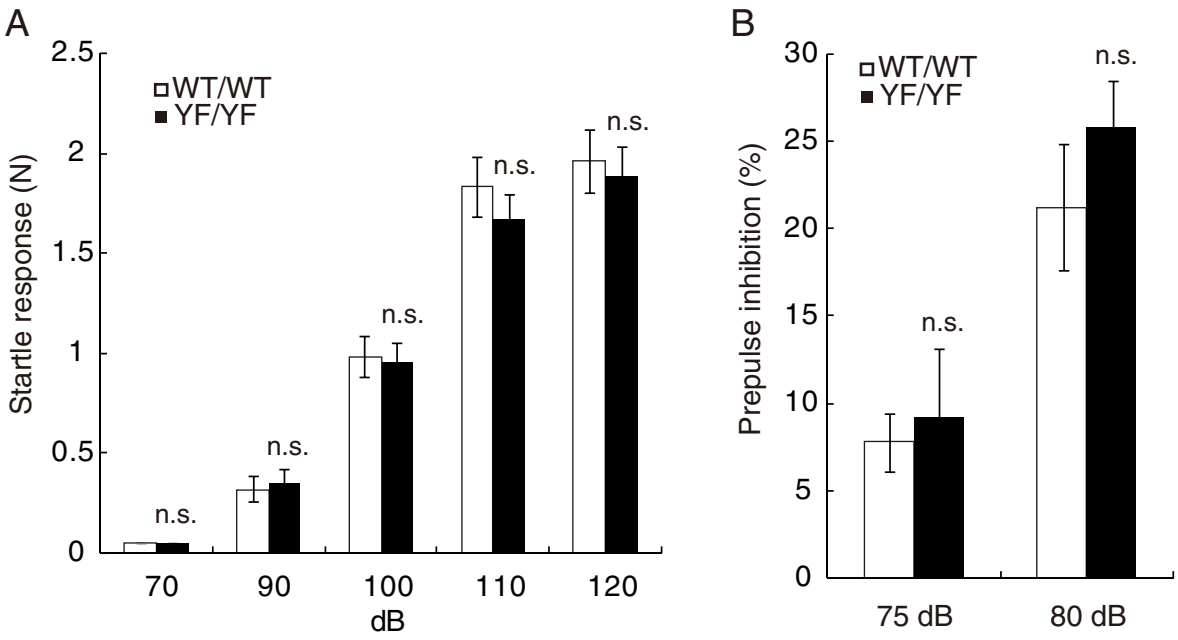
Supplementary Figure 9. Normal PKA kinase activity in the striatum of YF/YF mice. Striatal lysates from WT/WT and YF/YF mice were prepared and PKA activity in these lysates was measured with a PKA kinase activity assay kit (Assay designs). $p > 0.05$, $n = 4$, Student's *t* test. n.s.: not significant.



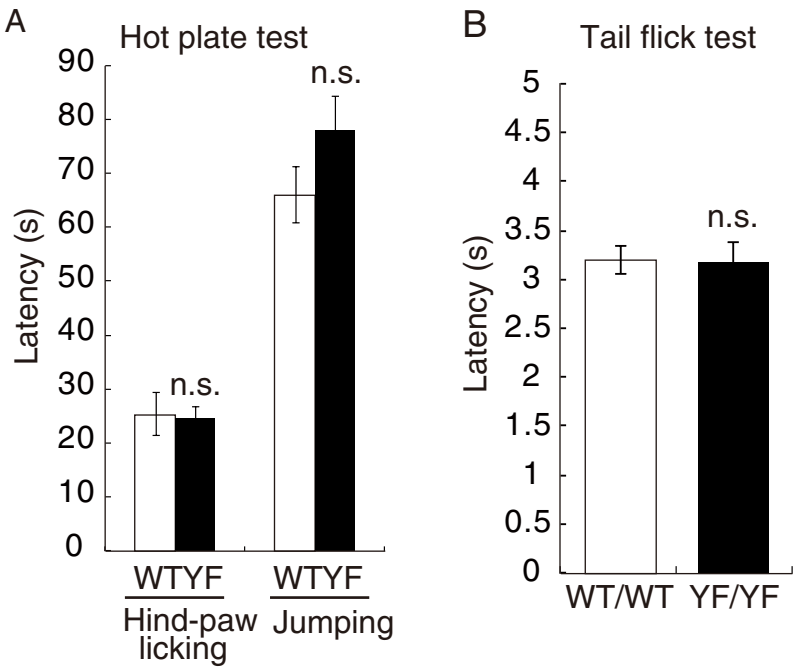
Supplementary Figure 1. Characterization of the rabbit anti-phospho-Tyr-1325 polyclonal antibody used in this study.



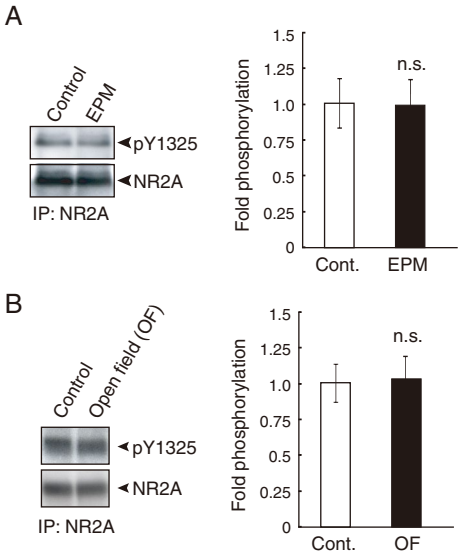
Supplementary Figure 2. Normal hippocampus- and amygdala-dependent learning in YF/YF mice**.**



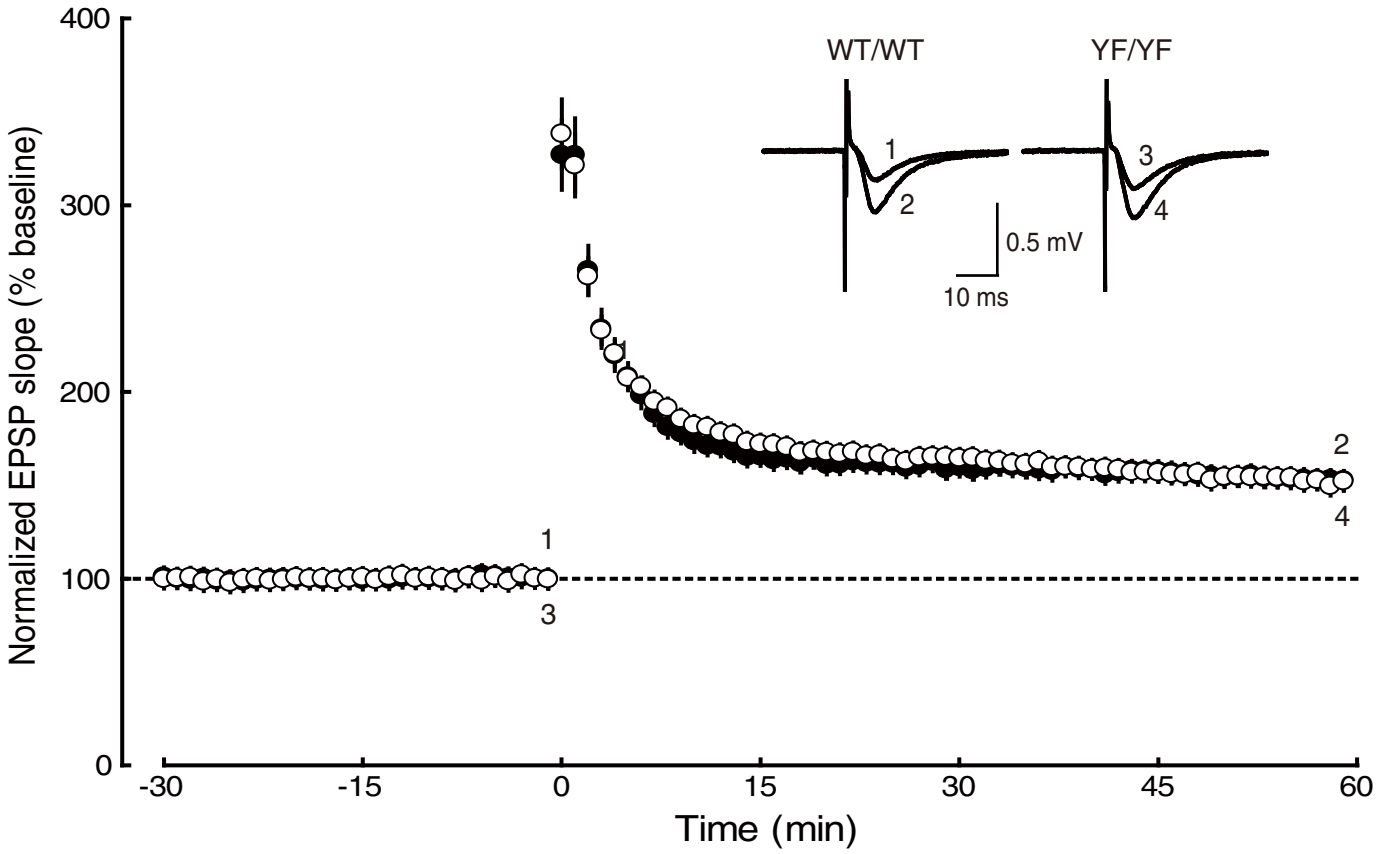
Supplementary Figure 3. Normal acoustic startle responses and pre-pulse inhibition in YF/YF mice.



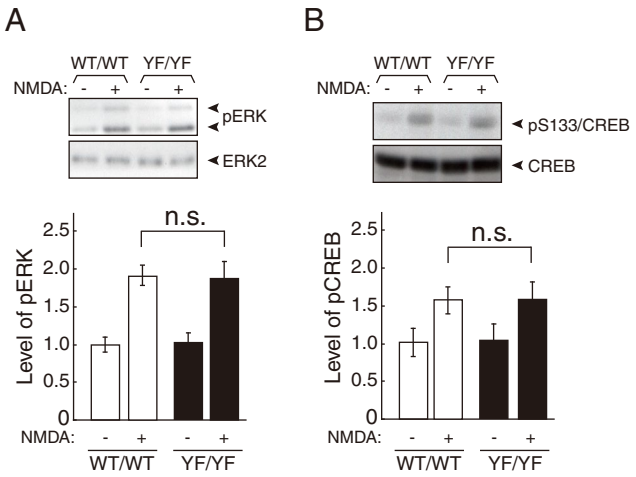
Supplementary Figure 4. Normal pain behavior in YF/YF mice.



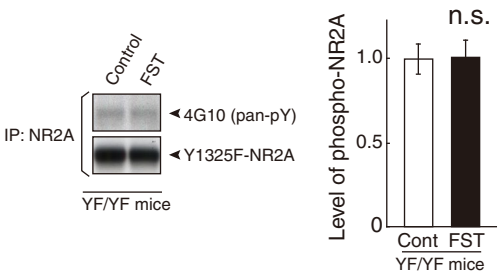
Supplementary Figure 5. Little effect of the performance of the elevated plus maze test (EPM) and the open field test (OF) on Tyr-1325 phosphorylation.



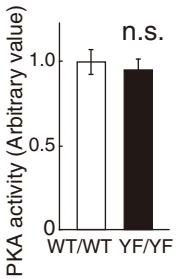
Supplementary Figure 6. Normal tetanus-induced long-term potentiation in YF/YF mice.



Supplementary Figure 7. Normal NMDA receptor-mediated signaling in the prefrontal cortex of YF/YF mice.



Supplementary Figure 8. Unchanged tyrosine phosphorylation of Y1325F-mutated NR2A during the forced swim test.



Supplementary Figure 9. Normal PKA activity in the striatum of YF/YF mice.