Neurobiology of Disease

Spred1 Is Required for Synaptic Plasticity and Hippocampus-Dependent Learning

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Germline mutations in *SPRED1*, a negative regulator of Ras, have been described in a neurofibromatosis type 1 (NF1)-like syndrome (NFLS) that included learning difficulties in some affected individuals. NFLS belongs to the group of phenotypically overlapping neuro-cardio-facial-cutaneous syndromes that are all caused by germ line mutations in genes of the Ras/mitogen-activated protein kinase extracellular signal-regulated kinase (ERK) pathway and that present with some degree of learning difficulties or mental retardation. We investigated hippocampus-dependent learning and memory as well as synaptic plasticity in *Spred1*$^{-/-}$ mice, an animal model of this newly discovered human syndrome. *Spred1*$^{-/-}$ mice show decreased learning and memory performance in the Morris water maze and visual-discrimination T-maze, but normal basic neuromotor and sensory abilities. Electrophysiological recordings on brain slices from these animals identified defects in short- and long-term synaptic hippocampal plasticity, including a disequilibrium between long-term potentiation (LTP) and long-term depression in CA1 region. Biochemical analysis, 4 h after LTP induction, demonstrated increased ERK-phosphorylation in *Spred1*$^{-/-}$ slices compared with those of wild-type littermates. This indicates that deficits in hippocampus-dependent learning and synaptic plasticity induced by *SPRED1* deficiency are related to hyperactivation of the Ras/ERK pathway.

Key words: Spred1; hippocampus; learning; Morris water maze; synaptic plasticity; LTP; LTD

Introduction

Sprouty-related proteins with an EVH-1 domain (Spred proteins) belong to a novel family of evolutionary conserved, negative regulators of the Ras/extracellular signal-regulated kinase (ERK) pathway (Casci et al., 1999; Wakioka et al., 2001). The Ras/ERK pathway is a signal transduction cascade that is crucially involved in various physiological and pathological processes, including the cellular mechanisms underlying synaptic plasticity and memory formation (English and Sweatt, 1996, 1997; Atkins et al., 1998; Selcher et al., 1999; Krab et al., 2008). Spred1 is a member of this protein family that is predominantly expressed in

adult mouse brain (Kato et al., 2003). Notably, heterozygous loss-of-function *SPRED1* mutations were recently shown to cause neurofibromatosis type 1 (NF1)-like syndrome (NFLS) in children that included learning difficulties (Brems et al., 2007), which suggests that this regulatory protein is involved in neural and behavioral plasticity as well. To test this hypothesis, we have studied hippocampus-dependent learning and memory with two different procedures in *Spred1*$^{-/-}$ mice and their heterozygous and wild-type littermates. In addition, we analyzed long-term potentiation (LTP) and depression (LTD) of synaptic responses in the hippocampal CA1 region of these mice, and examined Ras/ERK activation in relation to hippocampal synaptic plasticity.

Materials and Methods

Animals. *Spred1*$^{-/-}$ mice were generated by homologous recombination techniques. Exons containing the kit-binding domain and *Sprouty*-related cysteine rich domains were deleted (Inoue et al., 2005). Heterozygous mice were backcrossed 11 times onto a C57BL/6J background, and F11 heterozygous mice were then crossed to obtain three different genotypes (*Spred1*$^{+/+, +/-, -/-}$) that were used in all experiments, except in the T-maze where different genotypes were obtained by crossing F13 heterozygous mice. All mice were genotyped by means of PCR on DNA extracted from mice tails. Mice of mixed genotype were housed in groups of 3–5 at standard laboratory conditions (12 h dark/light cycle, constant room temperature and humidity, standard laboratory chow, and water *ad libitum*). Only female mice of similar age (maximum 4 weeks difference) were used for the experiments. Basic neuromotor tests and Morris

water maze were performed at the age of 3–5 months, T-maze at the age of 6–7 months, and LTP and LTD recordings at the age of 6–8 months. *Spred1*$^{-/-}$ mice are viable and show no changes in gross anatomy, except a shorter face and smaller size than their wild-type (WT) littermates (Inoue et al., 2005).

Neuromotor tests. Grip strength was measured using a device consisting of a T-shaped bar connected to a digital dynamometer (Ugo Basile). Mice were placed before the bar and when they grabbed it, they were gently pulled backwards until they released the bar. For each animal, 10 measurements were obtained. Motor coordination and equilibrium were tested on an accelerating rotarod (Med Associates). Mice were first trained to balance on a rotating rod for 2 min at constant speed (4 rpm). Four test trials with an intertial interval of 10 min followed, in which the rotarod accelerated from 4 to 40 rpm in 5 min. Time until they dropped from the rotarod was recorded (up to 5 min cutoff).

Pain sensitivity was tested in the tail-flick procedure. The end of the tail of the mouse (∼2 cm) was held in 50°C water, and time until the mouse withdrew its tail was measured. This experiment was performed with 12 WT, 12 *Spred1*$^{+/-}$, and 13 *Spred1*$^{-/-}$ female mice of the same genetic background as the ones used for the other experiments.

Evoked potentials. Evoked potentials were recorded to test the integrity of the visual and auditory afferent pathways. Visual evoked potentials (VEP) were registered on a Myos 4 plus digital EMG/EP recorder (Schwarzer). Mice were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg). An active needle electrode was placed between the eyes, a reference electrode at an occipital position (perpendicular to the active electrode), and a third electrode near the base of the tail (ground). Mice were adapted to the dark at least 30 min before starting the recordings. They were placed in front of a flash light and tracings were obtained by averaging 200 responses.

Brainstem auditory-evoked potentials (BAEPs) were recorded immediately after the VEP recording using the same equipment. A needle electrode was placed above each ear and referenced to a common electrode near the base of the tail. A fourth electrode, also placed near the base of the tail, was used as ground. Five-peak tracings were obtained by averaging 2000 responses evoked by 85–86 dB clicks emitted by a speaker placed 1 cm in front of the animal's head. Latencies of the first five peaks were measured. Three animals died during this study period (one WT, one *Spred1*$^{+/-}$, and one *Spred1*$^{-/-}$) and were excluded from further analysis.

Spatial learning. Hippocampus-dependent spatial learning was tested in WT ($n = 21$), *Spred1*$^{+/-}$ ($n = 21$), and *Spred1*$^{-/-}$ mice ($n = 16$) using the hidden-platform version of the Morris water maze. Two *Spred1*$^{-/-}$ animals died from unknown cause in the weeks after completion of the water maze; the data from these animals were excluded from further analyses. A 15 cm round platform was placed at a fixed position in a 150 cm circular pool, 1 cm beneath the surface of the water (26°C and opacified with nontoxic white paint). Each daily trial block consisted of four swimming trials (15 min intertrial interval), starting randomly from each of four starting positions. Mice that failed to find the platform within 2 min were guided to the platform. All mice had to remain on the platform for 15 s before they were returned to their cages. There were two sessions of five trial blocks followed by 2 d of rest and a probe trial. During these probe trials, the platform was removed from the pool, the mice were released in the opposite quadrant, and their search patterns were recorded for 100 s. Swimming paths of the mice during trial blocks and probe trials were recorded using EthoVision video tracking equipment and software (Noldus).

Visual discrimination learning. Hippocampus-dependent visual discrimination learning was tested in WT ($n = 20$), *Spred1*$^{+/-}$ ($n = 21$), and *Spred1*$^{-/-}$ ($n = 18$) animals using a T-shaped dry-land maze (arms: 50 cm long, 5 cm wide) as described previously (Van der Jeugd et al., 2008). One *Spred1*$^{-/-}$ mouse died during the training phase of the experiment and was excluded from further analyses. Each arm of the maze was closed by a sliding door located 10 cm from the junction. The mice were food deprived until 80–90% of their original body weight. During initial discrimination training, the mice were rewarded with a chocolate cereal to choose the arm with the white door and avoid the arm with the black door. A correct choice required the mouse to touch the chosen door

with its nose, after which the door was lifted to provide access to the reward placed behind it. The maze was wiped thoroughly between each trial to eliminate odor cues. The position of the rewarded (white) door was randomized between trials. Each mouse performed eight trials per day with a 5–10 min intertrial interval, and the first phase ended when the group average reached the criterion of ≥75% correct responses during three consecutive days of testing.

During the second phase, mice were trained to distinguish between two graphical images (5×5 cm^2, 50% white and 50% black surface) on the doors of the lateral arms. The different images (see also inserts in Fig. 2b) consisted of a black circle on a white background, a vertical black area on a white background, and a checkerboard pattern (they were designated stimulus A, B, and C in a randomized manner across subjects). During the first acquisition training, stimulus pair AB was presented, and A was rewarded. The sessions were repeated until the mice reached a score of 75% correct responses, or the performance was stable during three consecutive days. Next, mice were trained on stimulus pair BC, and B was rewarded. During the final mixed-trial training, two consecutive presentations of each pair (AB and BC) were given four times a day.

The concluding test phase comprised four trial blocks that consisted of three presentations of the trained pairs (AB and BC) and two presentations of an untrained transitive pair (AC). Correct responding to this transitive pair (i.e., choosing stimulus A) required transfer of the reward history of stimuli A and C obtained during the preceding learning on pairs AB and BC.

Brain slice recordings. *Spred1*$^{-/-}$ or WT female littermate mice were killed by cervical dislocation, and the hippocampus was rapidly removed and placed in carbogen-saturated (95% O$_2$/5% CO$_2$) ice-cold artificial CSF (ACSF). From the dorsal area of the right hippocampus, transverse 400-μm-thick slices were prepared with a tissue chopper and placed and maintained at 32°C in a submerged-type chamber, continuously perfused with ACSF (2 ml/min) saturated with carbogen. After ∼90 min incubation, one slice was arbitrarily selected and a lacquer-coated monopolar stainless steel or tungsten stimulation electrode was placed in the stratum radiatum. For recording, a glass electrode (filled with ACSF, 1–4 MΩ) was placed in the stratum pyramidale of area CA1 to record field EPSPs (fEPSPs). The time course of the fEPSP was inferred from its initial slope function. To assess basic properties of synaptic responses, input/output curves were recorded. The stimulation strength was adjusted to elicit a fEPSP slope of 35% of the maximum and kept constant throughout the experiment. Paired-pulse facilitation (PPF) was investigated by applying two pulses in rapid succession (interpulse intervals of 10, 20, 50, 100, 200, and 500 ms, respectively) at 120 s intervals. During baseline recording, three single stimuli (0.1 ms pulse width, 10 s interval) were measured every 5 min.

Conditioning stimuli for LTP and LTD were as follows. For LTP, a single theta-burst stimulation (TBS) was used: 10 pulses of four stimuli at 100 Hz, separated by 200 ms, 0.2 ms pulse width (Larson et al., 1986). For LTD, low-frequency stimulation (LFS) consisted of 1800 pulses at 2 Hz, with 0.2 ms pulse width delivered for 15 min. Immediately after TBS or LFS, evoked responses were monitored at 1, 4, 7, and 10 min and then subsequently recorded every 5 min up to 4 h. The recording of slices from mutant mice was interleaved by experiments with wild-type controls.

Western blot analysis. After completion of an LTP experiment, stimulated and unstimulated (time-matched control) slices were frozen in liquid nitrogen and stored at −70°C. The tissue from these slices and from contralateral hippocampi not used for electrophysiology was homogenized in ice-cold buffer containing 10 mM Tris HCl, pH 7.4, one Complete Mini Protease Inhibitor Cocktail tablet (Roche Diagnostics), and Protein Phosphatase Inhibitor Cocktails 1 and 2 (Sigma). Sample protein concentrations were established using the BCA protein assay kit (Pierce). Proteins were separated by NuPAGE NOVEX Bis-Tris gel 4–12% (Invitrogen) electrophoresis and transferred to a HyBond-P polyvinylidene difluoride (PVDF) membrane (GE Healthcare Biosciences). After blocking with 5% nonfat milk, membranes were incubated overnight at 4°C with polyclonal rabbit phospho-Erk1/2 (pErk1/2) antibody (Cell Signaling Technology), then incubated with horseradish peroxidase-conjugated anti-rabbit antibody (GE Healthcare Biosciences) for 1 h at room temperature, and visualized with enhanced
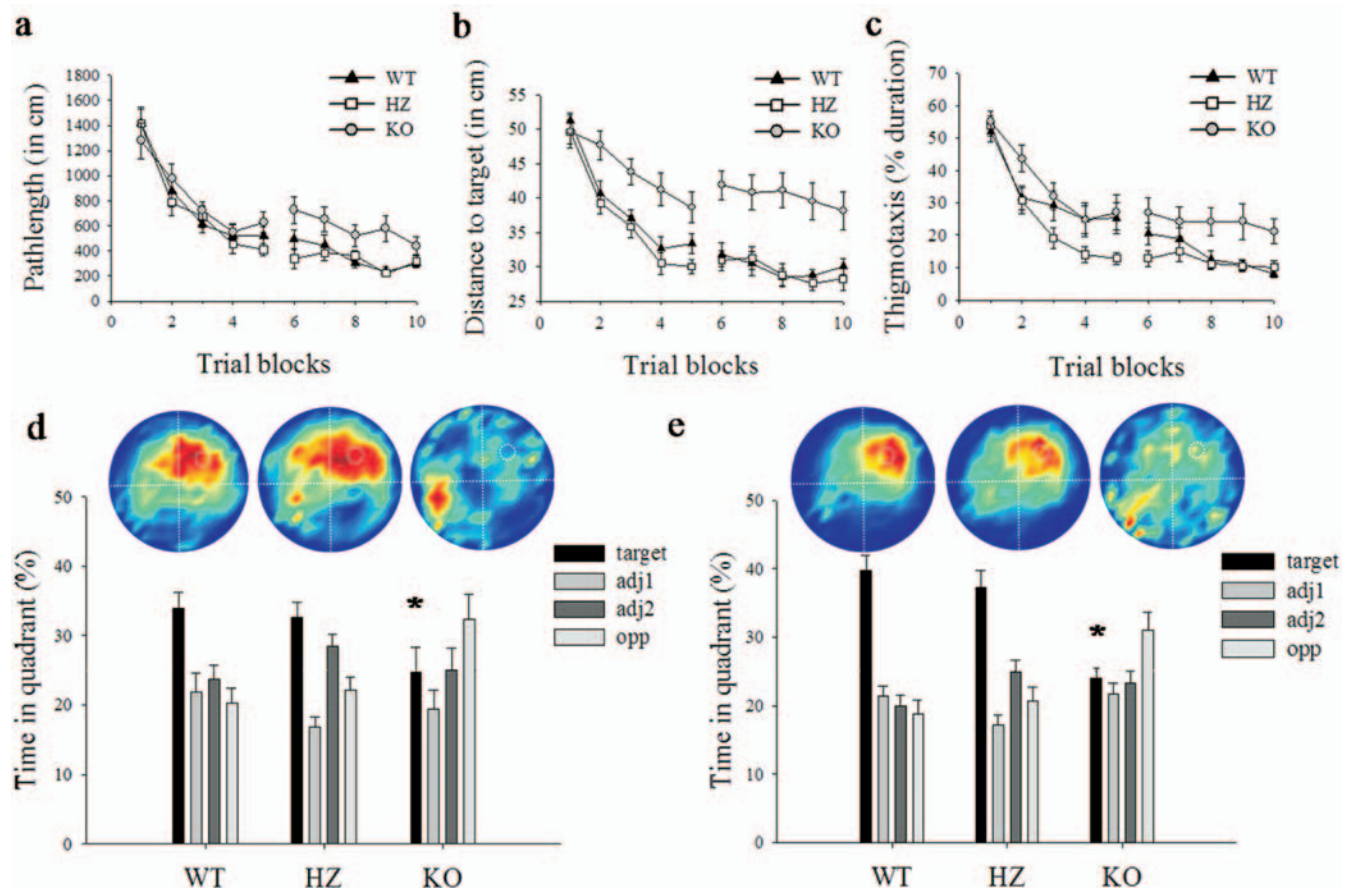
Figure 1.   Impaired Morris water maze performance in *Spred1*$^{-/-}$ (KO, n = 14) mice compared with wild-type (WT, n = 21) and *Spred1*$^{+/-}$ (HZ, n = 21) littermates. *a*, There is no significant effect of genotype on mean path length during the first week of acquisition training (p = 0.4), whereas *Spred1*$^{-/-}$ mice are unable to reach the same performance as the two other genotype groups during the second week (p < 0.001). *b*, *c*, More specific spatial performance measures indicate that (*b*) *Spred1*$^{-/-}$ mice display significantly longer distance to target from day 2 onwards (genotype × trial block interaction, p = 0.003), and (*c*) there is significantly more persistent thigmotaxic swimming in *Spred1*$^{-/-}$ mice compared with the other groups during the first (p = 0.016) and second acquisition week (p = 0.002). Also, spatial memory performance is impaired in *Spred1*$^{-/-}$ mice during the probe trials. *d*, The first probe trial already indicates that *Spred1*$^{-/-}$ animals spent less time searching the target quadrant than *Spred1*$^{+/-}$ or WT mice (p < 0.05). WT and *Spred1*$^{+/-}$ mice showed a significant preference for the target quadrant, whereas in *Spred1*$^{-/-}$ mice, there was no difference between time in the target quadrant and any of the other quadrants. This is also obvious from representative heat plots that are shown above the bar charts (dwell frequency is indicated by coloration from red through blue; circle indicates platform position). target, adj, opp indicate target, adjacent, and opposite quadrants, respectively. *e*, Results of the second probe trial demonstrate that WT and *Spred1*$^{+/-}$ further increased their preference for the target quadrant, whereas *Spred1*$^{-/-}$ mice failed to develop such a preference even after prolonged training (p < 0.001 for time spent in target quadrant).*p < 0.05 (pairwise comparison with WT values).

chemiluminescence (Perkin-Elmer). After stripping, membranes were reprobed with polyclonal rabbit Erk1/2 antibody (Cell Signaling Technology) and afterward with β-actin (Sigma).

   Quantification of dendritic arborization. Golgi-Cox staining of hippocampal sections obtained from *Spred1*$^{-/-}$ and wild-type littermates was performed according to the manufacturers' instructions using the FD Rapid GolgiStain Kit (FD NeuroTechnologies). Sagittal sections, 100 μm thick, were cut on a microtome with cryostat adaptations. Granule cell selection for further analysis was performed by a single observer, who was blind for genotype. Branching was manually counted using a 40× objective, and a calibration grid was used to count the number of spines per 10 μm.

   For transmission electron microscopy, mice were fixed by transcardiac perfusion with 6% glutardialdehyde in 0.1 M Sörensen phosphate buffer, postfixed in 1% OsO_4, dehydrated and embedded in Epon/glycid ether (Serva), using propylene oxide as an intermediate. Semithin and ultrathin sections were cut on a Reichert OmU 4 ultramicrotome at 1 μm and 70 nm, respectively, and the latter contrasted with uranyl acetate and lead citrate before evaluation in a Zeiss EM 910 transmission electron microscope.

   Statistics. All data are presented as mean and SEM. Differences between groups were examined using Mann–Whitney *U* test or ANOVA procedures with Fisher's least significant difference (LSD) test for *post*

hoc comparison. For within-group comparisons, Wilcoxon matched-pairs signed rank test was applied.

Results

Neuromotor performance and sensory perception

There were no differences in neuromotor and sensory perception abilities in *Spred1*$^{-/-}$ mice (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Both grip strength and accelerating rotarod performance were indistinguishable from WT. Visual as well as auditory evoked potentials were normal in appearance and peak latencies. Acute pain responses of *Spred1*$^{-/-}$ mice in the tail flick test were as vigorous as those of WT mice.

Spatial learning and memory

Hippocampus-dependent spatial learning was tested using the hidden-platform version of the Morris water maze. Two series of daily acquisition trial blocks were interspersed with two probe trials during which the escape platform was removed (Fig. 1). Mean path length decreased in all three genotypes during the acquisition trials, but by the end of training, *Spred1*$^{-/-}$ mice

failed to reach the same level of perfor-
mance as wild-type or heterozygous lit-
termates (Fig. 1a). While there was no
significant difference during the first
week of acquisition ($p = 0.380$), there
was a significant effect of genotype on
path length during the second week
($p < 0.001$).

Mean escape latency decreased in all
three genotypes during the acquisition tri-
als (supplemental Fig. 2a, available at
www.jneurosci.org as supplemental mate-
rial), but $Spred1^{-/-}$ mice were unable to
reach the same level of performance as
$Spred1^{+/-}$ or WT littermates (repeated
measures-ANOVA, $p < 0.001$). The longer
escape latency in the $Spred1^{-/-}$ group was
attributed to longer path lengths during
the final test days as mentioned above, as
well as to their lower swimming velocity
(supplemental Fig. 2b, available at www.
jneurosci.org as supplemental material).
The $Spred1^{-/-}$ group also showed a higher
tendency of floating behavior than
$Spred1^{+/-}$ and WT animals (supplemen-
tal Fig. 2c, available at www.jneurosci.org
as supplemental material).

However, differences in other spatial
performance measures, like mean dis-
tance to target and thigmotaxis, further
indicate that $Spred1^{-/-}$ mice have a de-
fect in spatial learning that is not reduc-
ible to motivational or sensory-motor
changes in these animals. All three genotypes showed a de-
crease in mean distance to target over the different acquisition
trials (Fig. 1b), but $Spred1^{-/-}$ mice displayed longer distance
to target from day 2 onwards (genotype × trial block interac-
tion, $p = 0.003$). $Spred1^{-/-}$ mice also displayed increased
thigmotaxis (i.e., percentage of time spent swimming along
the walls of the pool) in comparison with $Spred1^{+/-}$ and WT
littermates (Fig. 1c).

To determine whether or not $Spred1^{-/-}$ mice had actually
learned the spatial position of the platform, mice performed
probe trials with the platform removed (Fig. 1d–e). Notably,
$Spred1^{-/-}$ mice displayed a different search pattern from
$Spred1^{+/-}$ and WT mice during both probe trials (genotype ×
quadrant interaction, $p < 0.001$). In the first probe trial at day 8
(Fig. 1d), $Spred1^{-/-}$ animals spent less of their time in the target
quadrant ($p = 0.05$), and more time in the opposite quadrant
($p < 0.005$) compared with $Spred1^{+/-}$ and WT mice. They also
crossed the target area less frequently than $Spred1^{+/-}$ and WT
animals (data not shown). After prolonged training during the
second week of acquisition, differences in performance between
the different genotypes became even more explicit in the second
probe trial (Fig. 1e). Importantly, differences between genotypes
remained significant even when poor swimmers (swimming ve-
locity <5 cm/s during >50% of total swim time) were excluded
from analysis (supplemental Fig. 2d, available at www.
jneurosci.org as supplemental material), and detailed analysis of
search patterns failed to show any spatial preference in $Spred1^{-/-}$
animals during the last probe trial (supplemental Fig. 3, available
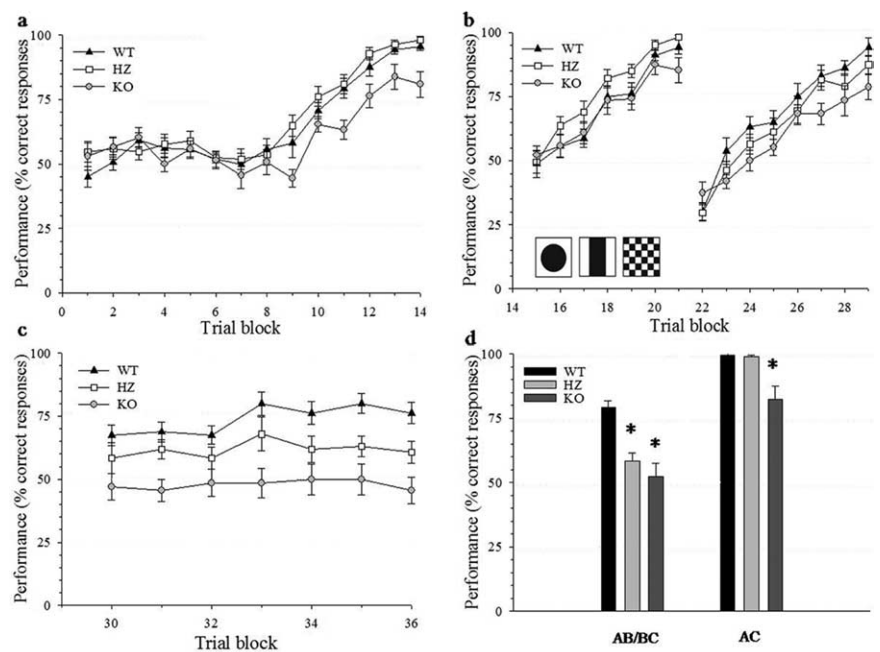at www.jneurosci.org as supplemental material).

Figure 2. Differences between $Spred1^{-/-}$ (KO, $n = 18$), $Spred1^{+/-}$ (HZ, $n = 21$), and WT ($n = 20$) mice in visual discrim-
ination learning and transitive task performance in the T maze. **a**, During initial black-white discrimination training, $Spred1^{-/-}$
mice showed less correct responses than their WT and $Spred1^{+/-}$ littermates, and fell behind their performance from trial block
10 onwards ($p = 0.005$). **b**, Acquisition training for AB and BC stimulus discrimination shows only borderline differences between
the genotypes (stimuli A, B, and C are depicted at the bottom of the graph). **c**, Performance on mixed trials revealed highly
significant differences between the genotypes ($p < 0.001$) with $Spred1^{+/-}$ animals displaying an intermediate level of perfor-
mance. **d**, During the final test trials, $Spred1^{-/-}$ and $Spred1^{+/-}$ mice were significantly less accurate in AB and BC discrimina-
tion compared with their WT littermates ($p < 0.001$). $Spred1^{-/-}$ mice also performed significantly worse than the two other
groups of mice on the untrained transitive pair AC ($p < 0.001$). *$p < 0.001$ (pairwise comparison with WT values).

Visual discrimination learning and transfer

The three $Spred1$ genotype groups were tested in a hippocampus-
dependent visual discrimination procedure and subsequent
transfer task using a T-shaped maze (Fig. 2). The tasks required
little motor proficiency, and performance measures related to
accuracy rather than latency or rate of responding. During the
first 14 trial blocks, all groups reached criterion and learned to
discriminate between black and white stimuli, but performance
was significantly influenced by genotype (repeated measures-
ANOVA, $p = 0.004$) and genotype × trial block interaction ($p =
0.005$). Actually, $Spred1^{-/-}$ mice failed to reach the same accu-
racy in black-white discrimination as $Spred1^{+/-}$ or WT litter-
mates, and fell behind their WT littermates from trial block 10
onwards (Fig. 2a). On the last day of training (trial block 14), an
average of 80.9% correct responses was observed in $Spred1^{-/-}$
mice compared with 98.2% in $Spred1^{+/-}$ and 95.6% in WT
animals.

During the second training phase (Fig. 2b), $Spred1^{-/-}$ mice
learned slower than their $Spred1^{+/-}$ and WT littermates, but the
differences did not reach significance (borderline genotype ef-
fects: $p = 0.054$ for pair AB, and $p = 0.079$ for pair BC; interaction
effects were not significant). Performance of $Spred1^{-/-}$ mice was
consistently lower than that of $Spred1^{+/-}$ and WT littermates on
the last trial blocks. There was a highly significant effect of geno-
type ($p < 0.001$) during the final mixed-trial presentations that
appeared to be much more difficult for the $Spred1^{-/-}$ mice than
for the other groups (Fig. 2c). Notably, performance of the het-
erozygous $Spred1^{+/-}$ mice was situated precisely between that of
$Spred1^{-/-}$ and WT mice, and post hoc comparisons revealed dif-
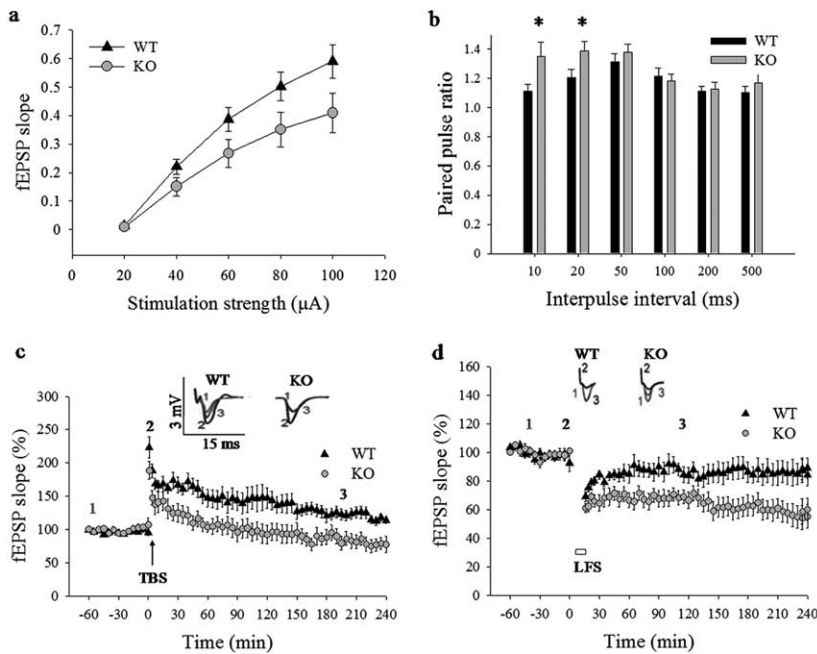ferences between WT and $Spred1^{-/-}$ mice ($p < 0.001$),

Figure 3. Differences in basic excitability and synaptic plasticity recorded on hippocampal slices from *Spred1*$^{-/-}$ (KO) mice and their WT littermates. ***a***, Input–output curves indicate progressively lower fEPSP slope at stronger stimulation in *Spred1*$^{-/-}$ ($n = 24$) than WT ($n = 31$) slices ($p = 0.049$). ***b***, PPF values (calculated as the ratio of the second on the first fEPSP slope) showed increased facilitation in *Spred1*$^{-/-}$ mice at 10 and 20 ms interpulse intervals ($n = 12$ for each genotype, $p < 0.05$, Mann–Whitney *U* test). ***c***, *Spred1*$^{-/-}$ mice showed a consistent impairment of LTP in CA1 area after TBS, with responses returning to baseline values after 55 min (12 slices, 6 animals for each genotype, $p < 0.05$, Wilcoxon matched-pairs signed rank test). Insets, Representative analog traces, numbers indicate trace-time point. ***d***, LTD is enhanced in *Spred1*$^{-/-}$ slices compared with those of WT mice ($n = 12$ slices, 6 animals for each genotype, $p < 0.05$, repeated-measures ANOVA). Open box under curves indicates duration of LFS. *$p < 0.05$ (pairwise comparison with WT values).

Spred1$^{+/-}$ and *Spred1*$^{-/-}$ mice ($p = 0.001$), and WT and *Spred1*$^{+/-}$ mice ($p = 0.003$). During this part of the procedure, percentage of correct responses averaged at 47.9% in *Spred1*$^{-/-}$ animals (i.e., chance level) compared with 61.7% in *Spred1*$^{+/-}$ and 73.8% in WT mice.

During the concluding test phase (Fig. 2*d*), *Spred1*$^{-/-}$, and to a somewhat lesser degree *Spred1*$^{+/-}$ mice, obtained a significantly lower level of accuracy than WT mice on the AB and BC pairs ($p < 0.001$). Also, *Spred1*$^{-/-}$ mice performed significantly worse than *Spred1*$^{+/-}$ and WT on the transitive AC pair ($p < 0.001$).

Hippocampal LTP and LTD
Field EPSPs (fEPSPs) were recorded in the CA1 region of hippocampal slices. Similar to what has been observed in *Nf1*$^{+/-}$ mice (Costa et al., 2002), *Spred1*$^{-/-}$ slices showed reduced fEPSP slopes, which was specifically pronounced at high stimulation strength ($p = 0.049$, ANOVA) (Fig. 3*a*). This was not caused by decreased excitability of the Schaffer collateral pathway as there was no significant difference in presynaptic fiber volley (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). PPF, a presynaptic form of short-term plasticity, was actually increased at 10 and 20 ms interpulse intervals in *Spred1*$^{-/-}$ compared with WT slices ($n = 12$, $p < 0.05$) (Fig. 3*b*), which was again similar to what has been observed in *Nf1*$^{+/-}$ mice (Costa et al., 2002). To examine the involvement of Spred1 in long-term synaptic plasticity, we compared hippocampal LTP in slices from *Spred1*$^{-/-}$ and WT littermates. A single TBS resulted in robust late-phase LTP (>4 h) in WT slices, whereas potentiation in *Spred1*$^{-/-}$ slices was decremental and returned to

baseline value after 55 min (both groups: $n = 6$; repeated-measures ANOVA, $p < 0.05$) (Fig. 3*c*). Finally, LFS induced LTD both in *Spred1*$^{-/-}$ and WT hippocampal slices (Fig. 3*d*), but the LFS train only produced transient response depression in WT slices, and returned to baseline after 90 min, while *Spred1*$^{-/-}$ slices showed robust LTD that lasted for >4 h (both groups: $n = 6$; $p < 0.05$).

Erk activation in hippocampus
We assayed Erk phosphorylation in hippocampi of *Spred1*$^{-/-}$ mice and their WT littermates. We could not detect a difference in hippocampal Erk phosphorylation pattern between *Spred1*$^{-/-}$ and WT mice in basal conditions (Fig. 4*b*). However, homogenates from hippocampal slices that had received the same TBS as were used for LTP induction did show small but significantly increased pErk2/Erk2 ratio in *Spred1*$^{-/-}$ compared with WT tissue ($p = 0.030$, ANOVA), which difference did not occur in time-matched control slices (Fig. 4*a–d*).

Dendritic arborization
To test whether the absence of Spred1 affected neuronal morphology, we used Golgi-Cox staining to assess spine density, and the number of proximal and distal dendrites of hippocampal granule cells. However, none of these measures showed significant differences between WT and *Spred1*$^{-/-}$ mice (supplemental Table 1 and supplemental Fig. 5, available at www.jneurosci.org as supplemental material).

Discussion
Germline mutations in *SPRED1*, a negative regulator of the Ras/ERK pathway, have recently been reported to cause NFLS (Brems et al., 2007). NFLS belongs to a group of phenotypically overlapping neuro-cardio-facial-cutaneous (NCFC) syndromes, which all present with a variable degree of learning difficulties or mental retardation. In NF1, visuo-spatial problems rank among the most common cognitive deficits (Hyman et al., 2005), and *Nf1* heterozygous mice consequently show impairments in visuo-spatial memory and hippocampal LTP (Costa et al., 2001, 2002). Genetic as well as pharmacological compensation for the loss of Ras regulation resulted in rescue of the learning defects in *Nf1*$^{+/-}$ mice (Costa et al., 2002). Moreover, the role of NF1 in learning and synaptic plasticity appears to be evolutionary conserved, because learning as well as cellular responses at the neuromuscular junction are impaired in *Drosophila* mutants of *Nf1* (Guo et al., 1997, 2000).

Also, in the newly described syndrome caused by *SPRED1* mutation, some, but not all, affected individuals display learning difficulties. The objective of this study was therefore to investigate hippocampus-dependent learning and memory, and synaptic plasticity in *Spred1*-deficient mice. We have demonstrated that *Spred1*$^{-/-}$ mice show clear defects in the spatial component of water maze learning, and in several phases of visual discrimination learning in the T maze, both measures for hippocampus-

dependent learning and neurocognitive functioning (Morris et al., 1982; O'Reilly and Rudy, 2001; Van der Jeugd et al., 2008). We also observed abnormalities in LTP and LTD of synaptic responses in hippocampal CA1 region (i.e., synaptic mechanisms essential for memory formation).

Synaptic plasticity in this hippocampal region is of crucial importance to spatial reference memory (Tsien et al., 1996). In Morris water maze, *Spred1*$^{-/-}$ animals performed significantly worse during acquisition training, but performance of *Spred1*$^{+/-}$ mice was not impaired. Changes in several spatial measures during acquisition training and in probe trial memory performance indicate that *Spred1*$^{-/-}$ mice have spatial learning and memory defects that might be attributed to impaired hippocampal functions. These effects of *Spred1* deficiency on hippocampus-dependent behaviors were further confirmed during visual discrimination training and transitive pair responding in the T maze. Notably, *Spred1*$^{-/-}$ mice showed marked impairments during nearly all stages of this elaborate procedure, but also heterozygous *Spred1*$^{+/-}$ animals performed at an intermediate level between that of their WT and *Spred1*$^{-/-}$ littermates, during the last and most difficult part of the T maze task. This latter finding suggests that heterozygous *Spred1*$^{+/-}$ mice may experience slight deficits in hippocampus-dependent cognitive functions as well.

In addition, we found Erk hyperactivation in *Spred1*$^{-/-}$ hippocampal slices, but only after LTP-inducing TBS. This observation indicates that *Spred1* is involved in hippocampal Erk pathways as well as in hippocampus-dependent neural and behavioral plasticity, which may be relevant for understanding human Ras/ERK disorders (Bentires-Alj et al., 2006; Krab et al., 2008). Increased Erk phosphorylation has also been shown in IL5-dependent cells transfected with a dominant negative *Spred1* mutant (Inoue et al., 2005), and in *SPRED1*$^{+/-}$ and $^{-/-}$ melanocytes stimulated with stem-cell factor (Brems et al., 2007).

In *Nf1*$^{+/-}$ mice, Erk hyperactivation was detected in both the hippocampus and the cortex. Notably, decreasing brain Ras/Erk activity in these latter mice rescued their LTP deficit, and reversed their spatial learning and attention impairments (Li et al., 2005). By and large, the behavioral deficits of *Spred1*$^{-/-}$ and *Spred1*$^{+/-}$ mice, as well as those of SPRED1 patients, mimic the neurocognitive impairments in mice and humans with germline loss-of-function mutations in *NF1* (Costa et al., 2002; Hyman et al., 2005; Brems et al., 2007). *Spred1*$^{-/-}$ mice also show a remarkably similar synaptic phenotype to *Nf1*$^{+/-}$ mice that includes impaired LTP, reduced fEPSPs at higher stimulation strength (but normal fiber volleys), and increased PPF at short PP intervals (Costa et al., 2002). Such behavioral and synaptic changes in *Nf1*$^{+/-}$ mice were attributed to increased neural inhibition and facilitated GABA release (Costa et al., 2002; Cui et al., 2008). However, this clearly contrasts with the enhanced learning, LTP, and glutamate release observed in mice with Ras overexpression in excitatory neurons (Kushner et al., 2005). Thus, the phenotype of mice with elevated Ras/Erk signaling (including *Spred1* and *Nf1* mutants)
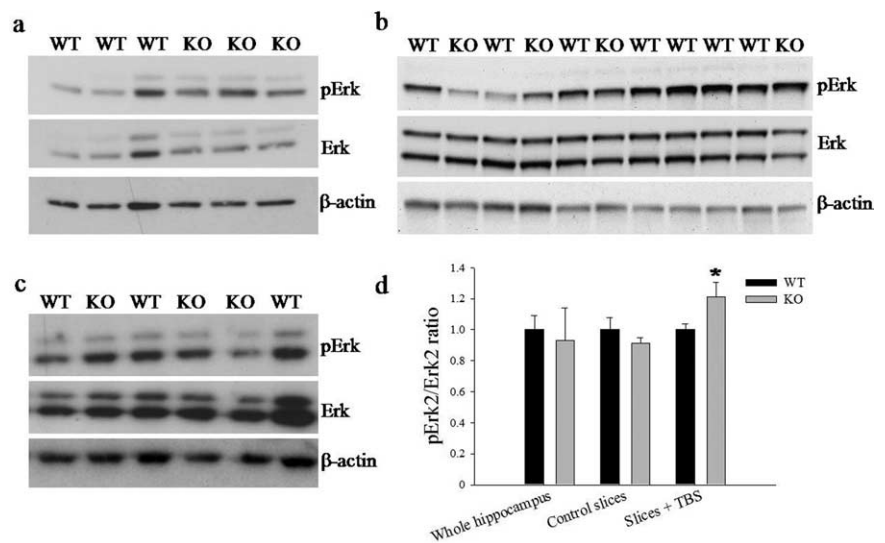
Figure 4. Differences in Erk activation between *Spred1*$^{-/-}$ (KO) and WT mice. ***a***, Western blots display enhanced Erk2 phosphorylation in *Spred1*$^{-/-}$ ($n = 5$) versus WT ($n = 8$) in TBS-stimulated hippocampal slices ($p = 0.030$, ANOVA). Three representative samples of each genotype are shown. β-Actin is shown as a loading control. ***b, c***, This enhanced pErk2/Erk2 ratio could not be found in whole hippocampi in basal conditions in two independent experiments ($p = 0.741$, $n = 7$ for WT, $n = 4$ for *Spred1*$^{-/-}$; $p = 0.888$, $n = 4$ for WT, $n = 5$ for *Spred1*$^{-/-}$) (***c***) nor in unstimulated control slices ($p = 0.339$, $n = 6$ for WT, $n = 6$ for *Spred1*$^{-/-}$). Three representative samples of each genotype are shown. ***d***, Bar-graphs indicate mean pErk2/Erk2 ratio which shows significantly higher ratios in the *Spred1*$^{-/-}$ slices (*$p = 0.030$, ANOVA) after TBS, but not in whole hippocampi or unstimulated slices.

could be related to increased neuronal inhibition, rather than to primary hyperexcitation.

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