Hippocampus-dependent learning and memory is impaired in mice lacking the Ras-guanine-nucleotide releasing factor 1 (Ras-GRF1)

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Abstract

Previous results have suggested that the Ras signaling pathway is involved in learning and memory. Ras is activated by nucleotide exchange factors, such as the calmodulin-activated guanine-nucleotide releasing factor 1 (Ras-GRF1). To test whether Ras-GRF1 is required for learning and memory, we inactivated the Ras-GRF1 gene in mice. These mutants performed normally in a rota-rod motor coordination task, and in two amygdala-dependent tasks (inhibitory avoidance and contextual conditioning). In contrast the mutants were impaired in three hippocampus-dependent learning tasks: contextual discrimination, the social transmission of food preferences, and the hidden-platform version of the Morris water maze. These studies indicate that Ras-GRF1 plays a role in hippocampal-dependent learning and memory. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Recent evidence suggests that MAP kinase signaling is involved in the formation of long-term memories. MAP kinase regulates the activity of the cAMP-responsive element-binding protein (CREB) (Impey et al., 1998), a transcription factor essential for long-term memory (Silva et al., 1998). Pharmacological experiments showed that MAP kinase is required for contextual (Atkins et al., 1998; Schafe et al., 1999) and spatial memory (Blum et al., 1999) as well as for long-term potentiation (LTP) (English and Sweatt, 1997), a candidate cellular mechanism of memory (Bliss and Collingridge, 1993). Since MAP kinase is regulated by Ras signaling, it is of interest to identify the components of the various Ras pathways, which contribute to learning and memory. Mutant mouse studies have implicated neuro-

fibromin (NF1), a GTPase activating protein that inactivates Ras, in learning and memory (Silva et al., 1997a).

Ras proteins cycle between active GTP-bound and inactive GDP-bound states (Boguski and McCormick, 1993). The activation of Ras is mediated by interactions with guanine-nucleotide exchange factors (GEFs). These proteins promote the release of GDP bound to Ras, allowing its replacement with activating GTP. At least two classes of Ras-specific exchange factors exist, the SOS and Ras-GRF families. Ras-GRF1 promotes the release of GDP bound to the Ras isoforms Ha-Ras (Jones and Jackson, 1998) and R-Ras (Gotoh et al., 1997) and this occurs through its C-terminal CDC25 domain. The Ras-GRF1-mediated activation of the Ras isoforms is regulated by Ca^{2+} signaling (Farnsworth et al., 1995; Buchsbaum et al., 1996; Fam et al., 1997) and also by G-protein-mediated signals (Shou et al., 1995; Mattingly and Macara, 1996; Zippel et al., 1996). In addition to activating Ras isoforms, Ras-GRF1 can also activate Rac, a family member of the Ras-related Rho GTPases (Innocenti et al., 1999) through a Dbl-like exchange domain in the central portion of the protein.

Ras-GRF1 is expressed almost exclusively in brain

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(Shou et al., 1992) and it is found primarily in postsynaptic densities, but not synaptic vesicles of mature neurons (Sturani et al., 1997; Zippel et al., 1997). In brain Ras-GRF2, a protein related to Ras-GRF1, has been identified (Fam et al., 1997). Ras-GRF2 has a similar domain structure to Ras-GRF1 and may therefore exert similar functions. Ras-GRF2 is also preferentially expressed in brain, however its tissue expression is not as restricted as Ras-GRF1 (Fam et al., 1997).

To determine whether Ras-GRF1-dependent signaling is required for learning and/or memory, we used gene targeting to inactivate the mouse gene encoding Ras-GRF1 on chromosome 9 (Gariboldi et al., 1994; Plass et al., 1996). The Ras-GRF1-deficient mice that we derived were suitable for studying learning and memory, since they did not suffer from obvious neurological abnormalities. The loss of Ras-GRF1 impaired hippocampus-dependent learning or memory, but spared amygdala-dependent learning and memory as well as motor learning. The presented results have been published in abstract form (Giese et al., 1997).

2. Methods

2.1. Generation of Ras-GRF1-deficient mice

For constructing the Ras-GRF1 targeting vector, a genomic clone was isolated from a 129/Sv library, and the position of an exon encoding part of the Dbl homology domain (Shou et al., 1995) was mapped. The 7 kb SacI fragment containing this exon was subcloned and a PGKneo cassette (Adra et al., 1987) was cloned into the AatII site of the exon (directly after codon 286) with the same transcriptional orientation as the Ras-GRF1 gene. R1 embryonic stem cells (Nagy et al., 1993) were transfected with the NotI-linearized Ras-GRF1 targeting construct. After selection with G418, 60 out of 260 clones were identified and characterized as proper targeted clones by Southern blot analyses (also no additional neo integrations were observed). Chimeras were generated by injection of cells from targeted clones into blastocysts. The male chimeras were mated with C57BL/6J females and heterozygous mutants deriving from one targeted clone were intercrossed to obtain homozygous mutants. The Cold Spring Harbor Laboratory animal facility is fully accredited by the American Association for the Accreditation of Laboratory Animal Care, and the animals are maintained in accordance with the Animal Welfare Act and the DHHS guide.

2.2. Behavioural studies

All behavioral studies were performed blind to the genotype of the animals. Animals homozygous for pink-eyed dilution were not studied because of their impaired vision. The results were tested with a one-way or two-way analysis of variance (ANOVA).

2.2.1. Rota-rod test

Eight Ras-GRF1-deficient and ten wild-type (WT) mice were tested on a rota-rod (Stoelting Instruments) with two trials per day (1 h intertrial interval) for 5 days. For a given trial, mice were placed on the rotating rod (3.5 rpm) and the rotation speed was accelerated constantly to 35 rpm within 5 min. The time to fall from the rod was measured.

2.2.2. Inhibitory avoidance test

Nine Ras-GRF1-deficient and nine WT mice were tested in an inhibitory avoidance test using a similar procedure to that previously published by Brambilla et al. (1997). The mice were tested in a chamber with light and dark compartments separated by a door. The light compartment was 16 cm wide and 12.1 cm long, and the dark compartment was 13.4 cm wide and 17 cm long. On the training day the mice were placed into the light compartment and the time to enter the dark compartment was measured. As soon as the mice entered the dark compartment the door was closed and two electrical shocks were delivered (0.4 mA for 2 s; intershock interval 5 s). After 24 h the mice were placed in the light compartment of the chamber and the time enter the dark compartment was recorded (3 min maximum). Since no mouse entered the dark compartment in less than 3 min, the mice were tested again (4 days later) with 10 min maximum testing time.

2.2.3. Contextual conditioning

Ten Ras-GRF1-deficient and ten WT mice were tested for contextual conditioning 24 h after conditioning as previously described (Bourtchuladze et al., 1994). Briefly, on the training day the mice were placed for 3 min into a conditioning chamber. After 2 min a tone (85 dB) was presented for 30 s and during the last 2 s of the tone an electrical shock (0.75 mA) was delivered. After 24 h the mice were placed into the conditioning chamber and freezing was scored for 5 min.

2.2.4. Contextual discrimination

Twenty Ras-GRF1-deficient and eighteen WT mice were tested in contextual discrimination as previously described (Frankland et al., 1998). In this task the mice were trained to discriminate between two chambers, one in which they were shocked and another in which they were not shocked. On day 1 the mice were pre-exposed to the two contexts for 10 min. On day 2 the mice were shocked in one context (paired): after 148 s a 2 s shock (0.75 mA) was delivered and the mice remained for another 30 s in the context. Additionally, the mice were exposed for 3 min to the other context (non-paired) during which time no shocks were delivered. On day 3 the
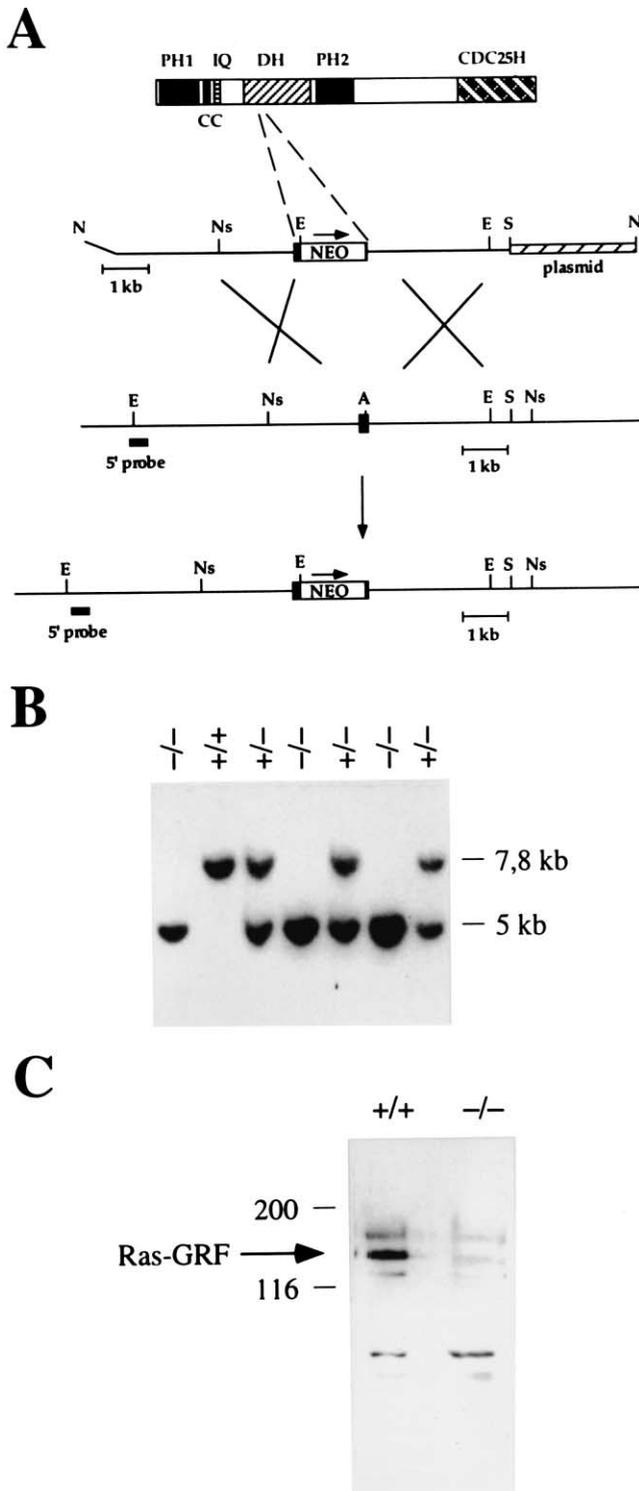
A

B

C

Fig. 1. Generation of Ras-GRF1-deficient mice. (A) The Ras-GRF1 targeting strategy, as well as the Ras-GRF1 protein and its domains are shown (PH, pleckstrin homology domain; CC, putative coiled coil; IQ, Ca^{2+}/calmodulin binding site; CDC25H, CDC25 homology domain). A neomycin gene (neo) was inserted into an exon encoding part of the Dbl homology domain. The location of the 5′-probe is indicated. Sites for the following restriction endonucleases are indicated: A, AatII; E, EcoRI; N, NotI; Ns, NsiI; S, SacI. (B) The Southern blot analysis strategy for genotyping is illustrated. Genomic DNA was digested with EcoRI and tested with the 5′-probe. −/−, homozygous; +/- heterozygous; +/+ WT. (C) Western blot analysis with protein from adult brain using an antibody recognizing the C-terminus of Ras-GRF1. In the homozygous mutants Ras-GRF1 (140 kDa) could not be detected.

2.2.5. Social transmission of food preference task

The Ras-GRF1-deficient mice were also tested in the social transmission of food preference task as previously described (Kogan et al., 1997). This test is based on the fact that mice develop a preference for foods that they recently smelled on the breath of other mice ('demonstrator' mice). Performance immediately after the interaction with the 'demonstrator' mice is not sensitive to hippocampal lesions. However, 24 h after the interaction with the 'demonstrator' mice, performance on this task is hippocampus-dependent (Bunsey and Eichenbaum, 1995). Seventeen Ras-GRF1-deficient mice and 11 control littermates were tested 21 h after the interaction with 'demonstrator' mice. In this 'delayed' test the mouse chow contained either cinnamon (1% per weight) or cocoa (2% per weight). Afterwards a randomly selected subset of the mice (12 Ras-GRF1-deficient and 9 WT mice) were tested with another pair of scents (marjoram, 2% per weight, and cumin 0.4% per weight). This time the mice were tested 30 min after the interaction with the 'demonstrator' mice ('immediate' testing).

2.2.6. Morris water maze

Seventeen Ras-GRF1-deficient and 15 WT mice were tested in the Morris water maze as previously described (Bourtchuladze et al., 1994). The mice were trained with two trials per day (1 min intertrial interval) for 14 days using a hidden platform. At the end of days 10 and 14 the mice were tested in a transfer test. During the probe trials the platform was removed and the mice were allowed to search for it for 60 s. After day 14 randomly selected subsets of mutants (n=8) and WT mice (n=7) were tested for one day (two trials) with a visible platform (for each trial in a different location).

3. Results

3.1. Generation of Ras-GRF1-deficient mice

The Ras-GRF1 targeting construct contained an insertion of a neomycin (neo) gene expression cassette in an

mice were shocked again in the 'paired' context and exposed to the 'non-paired' context. On day 4 the amount of freezing was measured for 3 min in each of the two contexts.

exon encoding part of the Dbl homology domain (Buchsbaum et al., 1996). The inserted neo gene has the same transcriptional orientation as the Ras-GRF1 gene (Fig. 1(A)). Embryonic stem (ES) cells were transfected with the linearized targeting construct and were tested for homologous recombination events by Southern blots. Cells from targeted ES cell clones were injected into blastocysts to generate chimeras. Homozygous mutants were obtained (Fig. 1(B)) in a Mendelian ratio ($n=285$; $\chi^2=0.62$; $P>0.70$), indicating that the introduced mutation is not lethal until the time of genotyping (postnatal day 21). Western blot analysis of protein from adult brain with antibodies raised against the C-terminus of Ras-GRF1, confirmed the loss of this protein in homozygous mutants (Fig. 1(C)). The antibodies did cross-react with some proteins, which appeared to be expressed at similar levels in the mutants and control littermates. A possible truncated protein (molecular weight of 108 kDa), starting from a methionine downstream of the neo insertion, and containing the CDC25 homology domain could not be detected in the mutants. Hence, we did not detect any Ras-GRF1 protein in homozygous mutants.

3.2. General description of Ras-GRF1-deficient mice

The Ras-GRF1-deficient mice appeared healthy and did not show any signs of neurological abnormalities (no ataxia, tremors, seizures, etc.). However, they had reduced body weight. At eight weeks of age the body weight of mutant males (21.6 ± 0.6 g) was reduced by approx. 23% in comparison to wild-type (WT) males (28.0 ± 0.4 g; one-way ANOVA: $F(1,14)=78.4$, $P<0.001$). The body weight of eight-week-old mutant females (19.3 ± 0.8 g) was reduced by approx. 10% in comparison to female WT littermates (21.4 ± 0.6 g; one-way ANOVA: $F(1,17)=4.49$, $P<0.05$). Light microscopy analysis of brain morphology did not detect any gross morphological deficits in the mutants (data not shown).

3.3. Normal motor coordination in Ras-GRF1-deficient mice

Abnormal motor coordination can be a confound for certain learning and memory tasks, such as the Morris water maze. To evaluate motor coordination in the Ras-GRF1-deficient mice, the mutants ($n=8$) and WT mice ($n=10$) were tested on an accelerating rota-rod for five days (Fig. 2(A)). Two-way ANOVA with repeated measures revealed no significant difference between mutant and WT mice ($F(1,16)=1.10$, $P=0.31$). Additionally, this analysis showed that both groups of mice improved with training ($F(4,64)=36.4$, $P<0.001$), and that there was no significant interaction between Genotype and Training ($F(4,64)=0.50$, $P=0.73$).
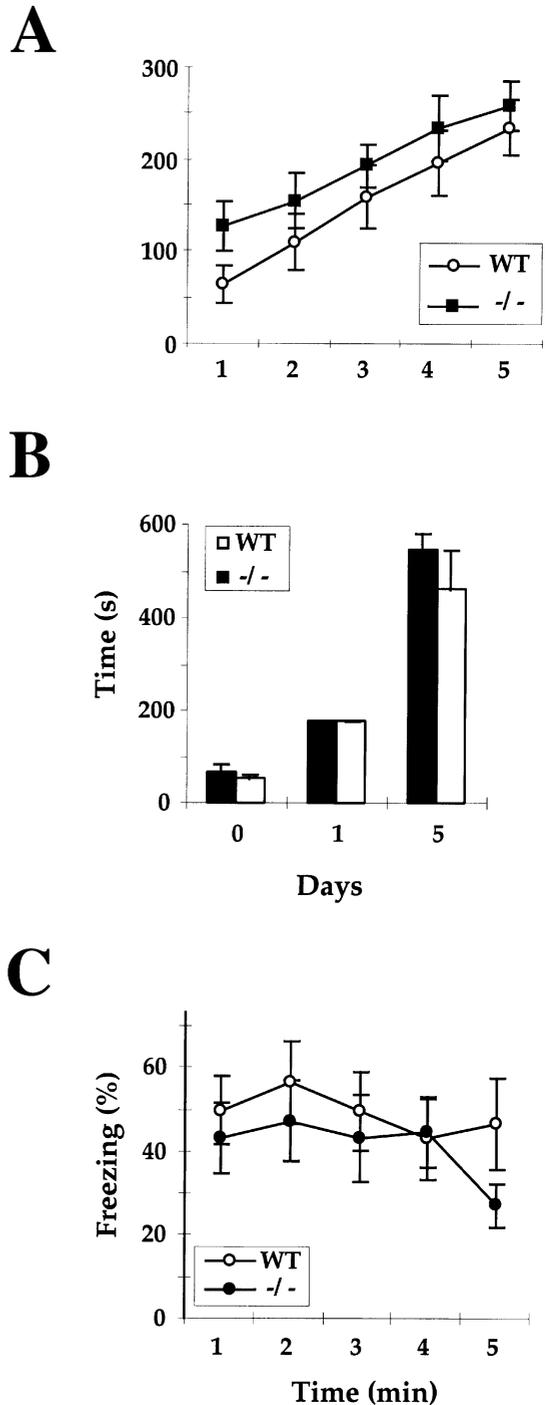

Fig. 2. Normal behaviors in Ras-GRF1-deficient mice. (A) In the rota-rod task there was no significant difference between Ras-GRF1-deficient and WT mice. The stay-time of the mice on an accelerating rotating rod is plotted versus the training day. (B) The Ras-GRF1-deficient and WT mice were indistinguishable in the inhibitory avoidance task. The time to enter the dark compartment is shown for the training day (day 0) and for days one and five after training. (C) The Ras-GRF1-deficient mice showed normal contextual conditioning. The conditioned response (%freezing) 24 h after training is shown.

3.4. Normal amygdala-dependent learning and memory in Ras-GRF1-deficient mice

3.4.1. Normal inhibitory avoidance of Ras-GRF1-deficient mice

To test amygdala-dependent learning and memory, Ras-GRF1 mutants ($n=9$) and WT mice ($n=9$) were tested in an inhibitory avoidance task (Cahill and McGaugh, 1990) (Fig. 2(B)). A two-way ANOVA with repeated measures revealed no significant differences between the mutants and WT mice tested five days after training ($F(1,16)=0.90$, $P=0.36$); Both groups showed a significant effect of training ($F(1,16)=101$, $P<0.001$). There was no significant interaction between genotype and training ($F(1,16)=0.63$, $P=0.44$). A planned comparison showed that both mutants and WT mice similarly avoided entering the dark compartment five days after training ($F(1,16)=0.80$, $P=0.38$).

3.4.2. Normal contextual conditioning in Ras-GRF1-deficient mice

Ras-GRF1-deficient ($n=10$) and WT mice ($n=10$) were tested in contextual conditioning, another task which requires intact amygdala function (Kim and Fanselow, 1992; Phillips and LeDoux, 1992) (Fig. 2(C)). Twenty-four hours after training freezing was tested during a 5 min interval. Two-way ANOVA with repeated measures showed that the mutants were indistinguishable from the WT mice (effect of genotype: $F(1,18)=0.60$, $P=0.44$; effect of test minute: $F(4,72)=1.62$, $P=0.18$; interaction genotype×test minute: $F(4,72)=0.76$, $P=0.55$).

3.5. Impaired hippocampus-dependent learning and memory in Ras-GRF1-deficient mice

3.5.1. Impaired contextual discrimination in Ras-GRF1-deficient mice

Contextual conditioning is thought to be both hippocampus and amygdala-dependent (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). However, recent studies have shown that pre-training hippocampal lesions have only a minor impact on contextual conditioning (Maren et al., 1997; Frankland et al., 1998; Cho et al., 1999). In contrast to contextual conditioning, contextual discrimination appears to be very sensitive even to pre-training hippocampal lesions (Frankland et al., 1998). Contextual discrimination tests the ability of mice to discriminate between two similar contexts, one in which they were shocked (paired) and another in which they were not shocked (non-paired) (Frankland et al., 1998). Ras-GRF1-deficient mice ($n=20$) and WT mice ($n=18$) were tested in contextual discrimination (Fig. 3). A planned comparison showed that WT mice froze more times in the 'paired' versus the 'non-paired' context ($F(1,34)=7.76$, $P<0.01$). The WT mice froze 71.9±3.5% of the testing time in the 'paired' context ver-
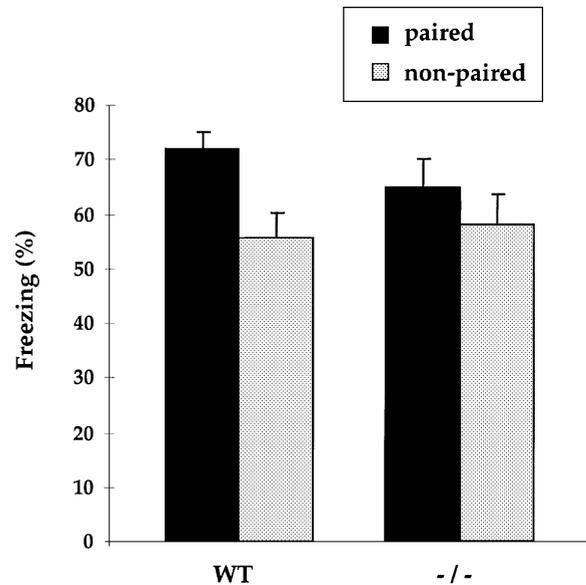

Fig. 3. Impaired contextual discrimination in Ras-GRF1-deficient mice. % Freezing to the 'paired' (with shock) and 'non-paired' context is shown. WT mice could discriminate between the two contexts ($P<0.01$), whereas the Ras-GRF1-deficient mice could not ($P=0.37$).

sus 55.7±4.7% in the 'non-paired' context. In contrast, the Ras-GRF1-deficient mice could not discriminate between the two contexts ($F(1,38)=0.83$, $P=0.37$). The mutants froze 65.1±5.4% of the testing time in the 'paired' context versus 58.1±6.0% in the 'non-paired' context.

3.5.2. Impaired social transmission of food preference in Ras-GRF1-deficient mice

We also tested the Ras-GRF1-deficient mice in the social transmission of food preference task (Bunsey and Eichenbaum, 1995) (Fig. 4). Planned comparisons showed that 21 h after interaction with the 'demonstrator' mice the Ras-GRF1-deficient mice ($n=17$) did not show any preference for the cued food ($F(1,32)=1.29$, $P=0.26$), whereas control littermates did ($n=11$; $F(1,20)=9.22$, $P<0.01$) (Fig. 4(A)). The mutants ate 0.34±0.05 g of cued food versus 0.27±0.04 g of non-cued food, and the WT mice ate 0.41±0.09 g of cued food versus 0.14±0.01 g of non-cued food. The total amount of eaten food, however, was the same for mutant and WT mice (mutants: 0.62±0.05 g, WT mice: 0.54±0.09 g; $F(1,26)=0.61$, $P=0.44$). In contrast to the 21 h-test, a one-way ANOVA showed that the Ras-GRF1-deficient mice ($n=12$) ate more cued than non-cued food when tested immediately after interaction with the 'demonstrator' mice ($F(1,22)=8.37$, $P<0.01$) (Fig. 4(B)). The mutants ate 0.54±0.08 g of cued food versus 0.27±0.04 g of non-cued food. The WT mice ($n=9$) ate 0.38±0.06 g of cued food versus 0.18±0.03 g of non-cued food ($F(1,16)=9.70$, $P<0.01$) (Fig. 4(B)). Thus, the Ras-GRF1 mutants performed normally when tested

A

After 21 Hours

- ■ cued
- ▨ non-cued

B

Immediate Testing

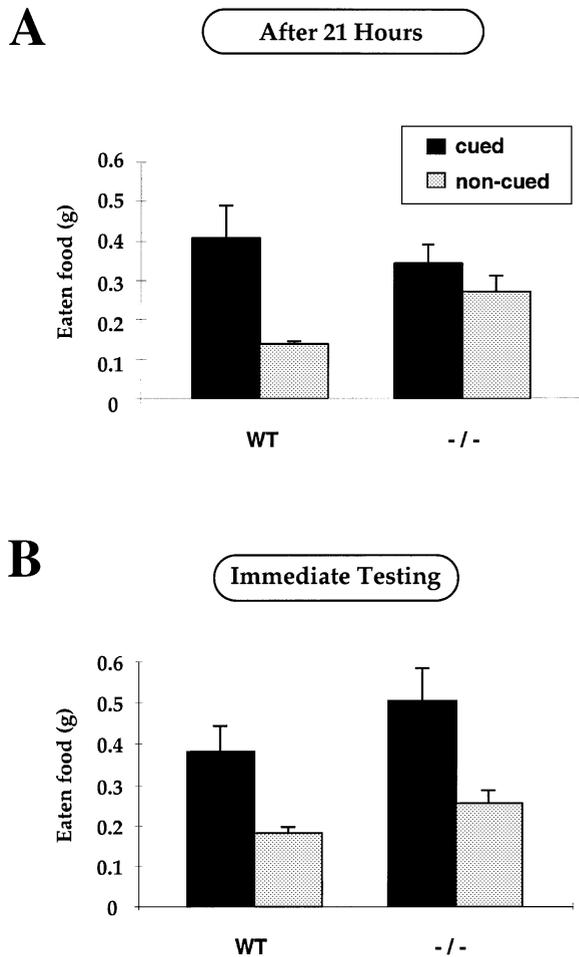Fig. 4.   Impaired social transmission of food preference in Ras-GRF1-deficient mice. (A) Twenty-one hours after the interaction with the 'demonstrator' mice the WT mice ate more cued than non-cued food ($P<0.01$), whereas the Ras-GRF1-deficient mice did not show any preference ($P=0.26$). (B) In contrast, in the 'immediate' test the Ras-GRF1-deficient mice ($P<0.01$) as well as WT mice ($P<0.01$) showed a preference for the cued food.

immediately after interacting with the 'demonstrators', but not 21 h later, a result consistent with the hypothesis that Ras-GRF1 mutants have abnormal hippocampal function.

3.5.3. Morris water maze impairments in Ras-GRF1-deficient mice

Performance in the hidden-platform version of the Morris water maze is also sensitive to hippocampal lesions (Morris et al., 1982; Cho et al., 1999). Ras-GRF1-deficient mice ($n=17$) and WT mice ($n=15$) were tested in this task with a distributed training protocol of two trials per day (Fig. 5). There was no significant difference in swimming speed between mutant and WT mice during the first training day ($F(1,30)=2.29$, $P=0.14$). Two-way ANOVA showed that the mutants needed more time than WT mice to reach the platform (effect of genotype: $F(1,30)=8.34$, $P<0.01$; effect of

training: $F(13,390)=20.20$, $P<0.001$; interaction genotype×training: $F(13,390)=0.97$, $P=0.48$) (Fig. 5(A)). Planned comparisons showed that the mutants needed more time to reach the platform at training days 3, 5, 8, 13, and 14 ($P's<0.05$).

In a transfer test given at the end of the hidden-platform training the mice were tested for spatial learning. Comparison of the search time in the target quadrant versus the search time in the other three quadrants showed that the mutants ($F(3,64)=8.45$, $P<0.001$) as well as the WT mice ($F(3,56)=18.28$, $P<0.001$) searched selectively during the transfer test (Fig. 5(B)). In contrast, two-way ANOVA showed that the mutants were impaired in platform crossings ($F(1,30)=15.9$, $P<0.001$). There was an effect of quadrant ($F(3,90)=9.29$, $P<0.001$) and no significant interaction between genotype and quadrant ($F(3,90)=1.41$, $P=0.24$). Further comparisons showed that the mutants crossed less frequently the exact former platform position than the WT mice ($F(1,30)=6.07$, $P<0.05$). The mutants crossed this location 2.1 ± 0.6 times, whereas the WT mice crossed it 3.8 ± 0.4 times. Furthermore, the mutants did not cross selectively the former platform position ($F(3,64)=1.78$, $P>0.10$), whereas WT mice did ($F(3,56)=7.04$, $P<0.001$) (Fig. 5(C)). It is important to note that the number of platform crossings is a more sensitive measure of probe trial performance than % search in the target quadrant. These results show that the mutants did not selectively search in the exact position where the platform was during training as much as controls, indicating that their searches were not as accurate as those of WT mice.

We also tested the mice (eight mutants and seven WT mice) in the visible platform task immediately after the hidden platform task (Fig. 5(A)). The results show that there was no significant difference between mutant and WT mice ($F(1,13)=3.03$, $P=0.11$). Surprisingly, the mutants appeared to find the visible platform faster than WT mice. These results suggest that the Ras-GRF1-deficient mice had the motivation, motor coordination and vision required to perform normally in the water maze. The increased escape latency, and the deficit in the transfer test indicate that the Ras-GRF1 mutation affects spatial learning.

4. Discussion

Previous results showed that a heterozygous null mutation of NF1, a Ras-GAP, affected spatial learning (Silva et al., 1997a) and contextual discrimination (Frankland et al., 1998), suggesting that the Ras signaling pathway is involved in hippocampus-dependent learning and memory. However, besides its GAP function, NF1 is known to play a role in other signaling pathways (Guo et al., 1997). To further probe whether the
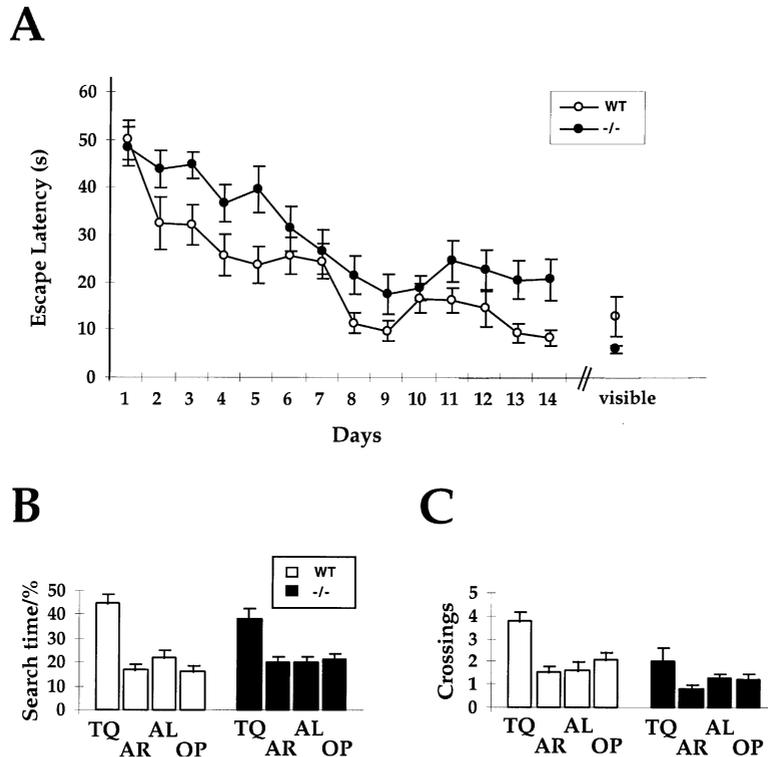
A

B

C

Fig. 5. Impaired spatial learning in Ras-GRF1-deficient mice. (A) The time to reach the platform in the water maze is plotted against training day. Planned comparisons showed that the Ras-GRF1-deficient mice needed more time to reach the platform than WT mice at days 3, 5, 8, 13, and 14. (B) There was no significant difference between mutant and WT mice in the % search time spent in the four quadrants (TQ: target quadrant, AR: quadrant adjacent right, AL: quadrant adjacent left, OP: quadrant opposite to target quadrant) during the transfer test given at the end of day 14. (C) In contrast, the Ras-GRF1-deficient mice crossed less frequently than WTs the exact site where the platform was during training ($P<0.05$). Furthermore, the WT, but not the Ras-GRF1-deficient mice, crossed the training platform position more often than equivalent sites in the other three quadrants ($P>0.10$).

Ras signaling pathway is involved in learning and memory, we disrupted the gene encoding the Ras activator Ras-GRF1 in mice. We chose this gene because its expression occurs primarily in mature neurons in brain (Shou et al., 1992; Zippel et al., 1997), suggesting that Ras-GRF1 plays only a minor role, if any, in developmental processes. Consistent with this idea, we did not find developmental abnormalities other than a reduction in body weight in the Ras-GRF1-deficient mice. The reduction in body weight is most likely due to a reduced level of growth hormone (Itier et al., 1998). The Ras-GRF1-deficient mice were impaired in three hippocampus-dependent learning and memory tasks: contextual discrimination, social transmission of food preferences, and the Morris water maze.

4.1. Abnormal hippocampal-dependent function in Ras-GRF1-mutant mice

Although the Ras-GRF1 mutant mice showed normal contextual conditioning, they were unable to discriminate between two similar contexts. Previous studies showed that pre-training hippocampal lesions do not affect contextual conditioning (Maren et al., 1997;

Frankland et al., 1998; Cho et al., 1999), but disrupt contextual discrimination (Frankland et al., 1998). Thus, these results suggest that the Ras-GRF1 mutation affected hippocampal function, but did not affect conditioned fear responses in the mutants. Similarly, the Ras-GRF1 mutation did not affect social transmission of food preferences immediately after the interaction with demonstrator mice, but disrupted it a day later. Importantly, while performance in this task 24 h after training is hippocampus-dependent, immediate performance is not (Bunsey and Eichenbaum, 1995). These results indicate that although the Ras-GRF1 mutation affected hippocampal function, it did not disrupt olfaction or social interactions. The water maze analysis of the Ras-GRF1 mutant mice are a further indication of impaired hippocampus-dependent learning and memory in the mutants. Performance in the hidden platform version of this test is hippocampal-dependent, while performance in the visible-platform test is not (Logue et al., 1997; Cho et al., 1999). In the hidden-platform task the mutants needed more time than control littermates to reach the platform than control littermates and in a transfer test at the end of training they did not search cross selectively for the platform location even though they searched selectively

in the target quadrant. Due to the lack of obvious performance abnormalities (for example, the mutants showed normal motor skills, normal performance in the visible platform test, etc), we interpret these findings as cognitive deficits in the mutants, in particular as a deficiency to learn accurately spatial information as accurately as controls. Together with the finding that the mutants performed normally in the visible platform test, these results indicate that the Ras-GRF1-mutant mice are deficient in hippocampal-dependent spatial learning. These spatial learning deficits in the Ras-GRF1 mutants, however, are relatively modest in comparison to spatial learning impairments in mutant mice, which lack LTP at the hippocampal CA1 synapse (e.g., Giese et al., 1998). Thus, it is possible that Ras-GRF1 is dispensable for LTP and that it is rather required for another cellular process, which also contributes to spatial learning. Thorough electrophysiological studies will test this hypothesis.

Interestingly, the mutant mice were not impaired in other forms of learning and memory. Motor learning appeared normal in the rota-rod task. Furthermore, normal contextual conditioning and normal inhibitory avoidance indicate that amygdala-dependent learning and memory was intact in the mutants. Altogether, the results presented here show that the loss of Ras-GRF1 affected specifically hippocampal function since it did not affect a number of other brain systems supporting a wide variety of behaviors, including conditioned fear and inhibitory avoidance, the olfactory and social responses required for immediate food preference, visible-platform learning in the water maze, motor coordination and motor learning.

4.2. Comparisons with other Ras-GRF1-deficient mouse lines

Recently, two other Ras-GRF1-deficient mouse lines were analyzed (Brambilla et al., 1997; Itier et al., 1998). Consistent with our Ras-GRF1 mutants Itier et al. (1998) described a body weight reduction for their mutants (Itier et al., 1998). In contrast Brambilla et al. (1997) did not observe body weight reductions for their mutants (Brambilla et al., 1997; but see, Orban et al., 1999). Furthermore, unlike our mutants, the Ras-GRF1[Brambilla] mutants showed normal spatial learning and impaired amygdala-dependent learning and memory (Brambilla et al., 1997). Thus, the phenotypes of our Ras-GRF1 mutants differ in several respects from the Ras-GRF1[Brambilla] mutants. First, our Ras-GRF1-deficient mice, but not the Ras-GRF1[Brambilla] mutants, had a reduction in body weight. Secondly, our Ras-GRF1-deficient mice, but not the Ras-GRF1[Brambilla] mutants were impaired in the Morris water maze. Thirdly, our Ras-GRF1-deficient mice did not show any impairments in inhibitory avoidance and contextual conditioning,

whereas analysis of the Ras-GRF1[Brambilla] mutants revealed memory impairments in these tasks (Brambilla et al., 1997). These results suggest that while our Ras-GRF1 mutation affected hippocampus-dependent learning and memory, the Ras-GRF1[Brambilla] mutation affected primarily amygdala-dependent learning and memory. Surprisingly, unlike our mutation (data not shown), the Ras-GRF1[Brambilla] mutation increased synaptic transmission in the hippocampal CA1 region (Brambilla et al., 1997).

What could account for the phenotypic differences between our and the Ras-GRF1[Brambilla] mice? First, neither mutation deleted the entire genomic DNA encoding the Ras-GRF1 gene. Therefore, it is possible that in one or both mutants the presence of partially active truncated protein products accounts for this difference. However, the immunoblot analyses carried out so far indicate that it is unlikely that either mutant has any such partial protein products (Brambilla et al., 1997).

Second, it is also possible that differences in the genetic background of the mutants studied may account for the phenotypic differences observed (Silva et al., 1997b). Unfortunately, the report describing the Ras-GRF1[Brambilla] mutants did not include a full description of the genetic background of the mutants studied (Brambilla et al., 1997), and therefore it is difficult to evaluate this possibility. It should be noted, however, that the body weight reduction of the Ras-GRF1[Itier] mutants was found found in two different genetic backgrounds (Itier et al., 1998).

Third, procedural differences between the behavioral tasks used to study the our and the Ras-GRF1[Brambilla] mutants could account for their apparent phenotypic differences. For example, water maze impairments may not have been detected in the Ras-GRF1[Brambilla] mutants, because these mice were tested with an intensive training protocol (six trials per day) that could have masked their spatial learning deficits. Furthermore, there are no published reports of contextual discrimination or social transmission of food preference studies for the Ras-GRF1[Brambilla] mutants. Therefore, it is possible that just as our mutants, the Ras-GRF1[Brambilla] mutants may also have impairments in these hippocampus-dependent tasks. However, it is unlikely that procedural differences explain the differences concerning body weight, inhibitory avoidance, contextual conditioning and hippocampal synaptic transmission since very similar protocols were used.

Fourth, it is possible that the different neo gene insertions in the two Ras-GRF1 mutants account for their phenotypic differences. Recently, it has been suggested that the neo gene can affect the expression of nearby genes (Olson et al., 1996; Pham et al., 1996; Taylor et al., 1998). While we inserted a neo gene into an exon encoding part of the Dbl homology domain in the same transcriptional orientation as the Ras-GRF1 gene, Brambilla et al. (1997) replaced exons encoding the N-ter-

minal region of the CDC25 homology domain with a neo gene inserted in the opposite transcriptional orientation (Brambilla et al., 1997). Itier et al. (1998) introduced a deletion in an exon coding for the CDC25 homology domain (Itier et al., 1998). However, the precise nature of this mutation is not described. Since our Ras-GRF1-deficient mice have a similar body weight reduction as the Ras-GRF1[Itier] mutants (these mutants have not been tested for learning/memory), it is likely that in the Ras-GRF1[Brambilla] mutants the expression of genes adjacent to Ras-GRF1 is altered by the neo gene insertion. It is important to note that the possible causes listed above could have affected the locus most affected by the mutation (in our case not the amygdala, but the hippocampus).

These studies suggest that the strategy for gene disruption can affect the phenotype of mutant mice. Partial protein products could have a function, and the neo gene could affect the expression of nearby genes. However, these potential confounds can be avoided in future studies. It is possible to delete the neo gene with the Cre/loxP technology (Sauer, 1993; e.g., Giese et al., 1998), and extensive immunoblot analysis can determine whether the mutation resulted in truncated proteins. These issues are specially important for gene products of unknown function. However, these concerns are not as critical when the phenotype of a mutant is consistent with previous studies with other molecular perturbation techniques, such as pharmacology and antisense technology.

4.3. Conclusion

Despite their phenotypic differences, both Ras-GRF1 mutants specifically affected learning and memory. Together with previous findings, these results suggest that the Ras signaling pathway is involved in learning and memory. The Cre/loxP recombination system allows the generation of region-restricted null mutations and the restriction of the Ras-GRF1 null mutation to brain areas will characterize further the role of this important molecule for learning and memory.

Acknowledgements

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