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Cognitive impairments in adult mice with constitutive inactivation of RIP140 gene expression

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ABSTRACT

Receptor-interacting protein 140 (RIP140) is a negative transcriptional coregulator of nuclear receptors such as estrogen, retinoic acid or glucocorticoid receptors. Recruitment of RIP140 results in an inhibition of target gene expression through different repressive domains interacting with histone deacetylases or C-terminal binding proteins. In this study, we analyzed the role of RIP140 activity in memory processes using RIP140-deficient transgenic mice. Although the RIP140 protein was clearly expressed in the brain (cortical and hippocampus areas), the morphological examination of RIP140^{-/-} mouse brain failed to show grossly observable alterations. Using male 2-month old RIP140^{-/-}, RIP140^{+/-} or RIP140^{+/+} mice, we did not observe any significant differences in the open-field test, rotarod test and in terms of spontaneous alternation in the Y-maze. By contrast, RIP140^{-/-} mice showed long-term memory deficits, with an absence of decrease in escape latencies when animals were tested using a fixed platform position procedure in the water-maze and in the passive avoidance test. Noteworthy, RIP140^{-/-} mice showed decreased swimming speed, suggesting swimming alterations that may in part account for the marked alterations measured in the water-maze. Moreover, RIP140^{+/-} and RIP140^{-/-} mice showed a significant increase in immobility time in the forced swimming test as compared with wild-type animals. These observations revealed that RIP140 gene depletion results in learning and memory deficits as well as stress response, bringing to light a major role for this transcriptional coregulator in the neurophysiological developmental mechanisms underlying cognitive functions.

INTRODUCTION

Nuclear receptors (NR) are ligand-activated transcription factors that bind to specific responsive elements located in the regulatory regions of target gene promoters and recruit various transcriptional coregulators resulting in transcription activation (coactivators) or repression (corepressors). (Ordóñez-Morán & Muñoz, 2009). Receptor-interacting protein 140 (RIP140), also named nuclear receptor interacting protein-1 (NRIP1), is a transcriptional coregulator of NR first described as recruited by the estrogen receptors (ER α) (Cavaillès *et al.* 1995). RIP140 interacts with several members of the NR superfamily including glucocorticoid receptors, thyroid hormone receptor, and retinoic acid receptors RAR/RXR (L'Horset *et al.* 1996; Lee *et al.* 1998; Augereau *et al.* 2006). RIP140 mainly exerts a negative transcriptional activity mediated by four distinct repression domains acting in part through the recruitment of class I and II histone deacetylases (HDAC) and C-terminal binding-proteins (CtBP) (Wei *et al.* 2000; Vo *et al.* 2001 Castet *et al.* 2004; Augereau *et al.* 2006). Moreover, RIP140 also exerts its repressive activity by a competition with transcriptional coactivators. For example, evidence was provided for a competition between RIP140 and the transcriptional coactivators, SRC-1 and p300/CBP-associated factor (PCAF) for recruitment by nuclear receptor (Treuter *et al.* 1998; Chen *et al.* 2004; Hu *et al.* 2004). PCAF is a transcriptional cofactor that bears an intrinsic acetyltransferase activity, remodeling chromatin structure towards a more decompacted state associated to transcriptional activation. We recently analyzed the role of PCAF in learning and memory process (Maurice *et al.* 2008) using PCAF knockout (KO) mice. We showed that PCAF is involved lifelong in the chromatin remodeling mechanisms needed for memory formation and response to stress. By competing with PCAF at the transcriptional level, RIP140 might therefore participate in physiological regulation of cognitive processes.

Additional indirect evidence can be put forward for RIP140 enrolment in the modulation of NR transcriptional activities sustaining cognitive plasticity. For instance, hippocampal ER α has been involved in memory formation (Fugger *et al.* 1998, 2000; Sanchez-Andrade & Kendrick, 2011) whereas glucocorticoids impacted learning and memory, particularly through aversive stimuli-reinforced pathways (Kolber *et al.* 2008; for review, see Roozendaal, 2000). RIP140 may therefore be recruited during the learning and/or consolidation processes sustaining hippocampal-dependent

memory formation. Finally, the gene coding for RIP140 being located on chromosome 21 (Katsanis *et al.* 1998), an increased expression of the RIP140 protein has been demonstrated in hippocampus of patients with Down's syndrome who exhibit strong cognitive disabilities (Gardiner, 2006). Altogether, these observations support a putative role of RIP140 in the regulation of gene expression related to cognition.

In order to determine whether RIP140 activity is involved in learning and memory function, we performed the behavioral phenotyping of RIP140^{+/+}, RIP140^{+/-} and RIP140^{-/-} mice. Two months-old male animals were analyzed in behavioral procedures assessing general activity, short-term and long-term, spatial and non-spatial memory as well as behavioral despair. Our data uncover for the first time a major role of RIP140 in the central nervous system with an implication in the control of processes involved in memory formation and response to stress.

MATERIALS AND METHODS

Mouse breeding

The generation of RIP140 KO mice at the Imperial College (London) has been previously described (White *et al.* 2000). Mice were backcrossed during six generations on a C57BL/6J background. RIP140^{-/-}, RIP140^{+/-} and RIP140^{+/+} C57BL/6J wild-type (WT) mice were propagated by heterozygous breeding (at least 5 generations) at the IRCM animal facility (Montpellier). Animals were housed in group, allowed food and water *ad libitum* except during experiments. They were maintained in a controlled environment (22 ± 1 °C, 55 ± 5% humidity) with a 12 h:12 h light:dark cycle, lights on at 7:00 h. All experiments have been performed on 2-month old +/+, +/- and -/- male littermates. Behavioral testing was performed between 10:00 to 17:00 h. All animal procedures were conducted in strict adherence of European Union Directive of 24 November 1986 (86-609).

Histological studies

Mice were killed in a euthanasia cabinet filled with carbon dioxide for 1 min. Brains were quickly removed after animal decapitation using a caudal approach. Brains were then post-fixed in 4% paraformaldehyde and paraffin-embedded. Thick sections (5 µm) were stained with haematoxylin-eosin or cresyl violet and luxol fast blue (Merck Chemicals, Nottingham, UK) or incubated in citrate buffer solution for immunofluorescence. For the luxol fast blue staining, slices were incubated overnight at 56°C in a luxol fast blue solution and differentiated in lithium carbonate solution. Slides were counterstained with a cresyl violet solution and mounted for microscopy. For immunofluorescence, the sections were incubated in phosphate buffer saline with 5% normal goat serum for 3 h to reduce non-specific binding and then incubated overnight at 4°C with the anti-RIP140 antibody (Abcam, Cambridge, UK) diluted in phosphate buffer saline containing 1% BSA. After washing, the sections were incubated with the secondary antibody, Alexa488-conjugated anti-rabbit goat antibody (Invitrogen, Cergy-Pontoise, France), for 1 h at room temperature, counterstained with Hoechst and mounted for fluorescence microscopy.

β-galactosidase staining

Brains were fixed in 2.5% methanol-free paraformaldehyde during 1 h at 4 °C. After three washes in cold phosphate buffer saline with 0.2 mM MgCl₂, 0.02% NP-40 and 0.01% sodium deoxycholate, tissues were incubated in a LacZ staining solution (10 mM K₃Fe(CN)₆, 10 mM K₄Fe(CN)₆, 3 H₂O and 1.5 mg/ml X-Gal) overnight at 37°C protected from light and post-fixed in 4% methanol-free paraformaldehyde.

Behavioral studies

Open-field behavior

The general mobility of mice was first examined using an open-field procedure. Two concentric circles (Ø 15 cm and 45 cm) were drawn on the floor of a circular wooden arena (Ø 75 cm), with the outer ring being divided into 8 partitions and the middle ring into 4 partitions. The open-field session consisted of placing the mouse in the center circle and monitoring its movements for 10 min using a video camera (Maurice *et al.* 2008). The following parameters were evaluated manually *ex tempore* by an experienced experimenter: (1) the time taken to move out of the center circle; (2) locomotion activity, in terms of distance traveled (m) calculated from the number of partitions crossed; (3) immobility duration; (4) locomotion speed, calculated as total distance over time in movement (total session time minus immobility and latency to start the exploration); (5) locomotion activity in the five central partitions; (6) number of rearing behaviors; (7) number of grooming behaviors; and (8) number of defecations.

Rotarod test

The motor coordination was measured in the rotarod test. The animals were first trained to walk forward on the rotating axis (20 rpm, constant speed) of a rotarod apparatus (Model 4700, Ugo Basile, Comerio, Italy). Training was stopped when the mouse stayed at least 30 sec on the axis without falling down. This pre-training is a rapid procedure to insure that the animals understand the requested motor response (back-pedalling to avoid falling), while reinforcing the validity of the measure with a minimal impact. 30 min later, the animals were replaced on the rotating axis and the latency to fall was measured with a cut-off at 600 sec. 24 h later, the same manipulations were repeated and performances averaged per mouse.

Spontaneous alternation in the Y-maze

The maze was made of grey polyvinylchloride. Each arm was 40 cm long, 13 cm high, 3 cm wide at the bottom, 10 cm wide at the top, and converging at an equal angle. Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8 min session. The series of arm entries, including possible returns into the same arm, was recorded visually. An alternation was defined as entries into all three arms on consecutive occasions. The number of maximum alternations was therefore the total number of arm entries minus two and the percentage of alternation was calculated as (actual alternations/maximum alternations) x 100.

Step-down type passive avoidance test

Long-term non-spatial memory was evaluated using the step-down type passive avoidance procedure. The apparatus consisted of a transparent acrylic cage (30 x 30 x 40 cm high, light 15 W) with a grid-floor, inserted in a soundproof outer box (35 x 35 x 90 cm high). A wooden platform (4 x 4 x 4 cm) was fixed at the centre of the grid-floor. Electric shocks (1 Hz, 500 ms, 43 V DC) were delivered to the grid-floor using an isolated pulse stimulator (Model 2100, AM Systems, Everett, WA, USA). The training involved two separate sessions, at 90-min time interval. Each mouse was placed on the platform and shocks were delivered for 15 s when it stepped down and placed its four paws on the grid-floor. Step-down latency, as well as the numbers of vocalizations and flinching reactions were measured. Shock sensitivity was evaluated by summing these last two numbers. Animals which did not step down within 60 s during the second session were considered as remembering the task and taken off without receiving more shocks. The retention session was carried out 24 h after the first training, and performed similarly as training but with no shock: each mouse was placed again on the platform and the step-down latency was recorded with a cut-off of 300 s. Two measures of retention were analyzed: the step-down latency and the number of animals reaching an avoidance criterion, defined as correct if the retention latency was higher than 3-fold the second training latency and at least greater than 60 s. Basically, the median latency could be considered as a qualitative index of memory capacities, whereas the percentage of animals to criterion could be considered as a quantitative index (Maurice *et al.* 1998; Phan *et al.* 2002).

Place learning in the water-maze

Spatial learning was analyzed using a water-maze procedure. A transparent Plexiglas non-slippery platform (Ø 10 cm) was immersed in a circular pool (Ø 170 cm, height 40 cm), under the water surface during acquisition. The water temperature, $23 \pm 1^\circ\text{C}$, light intensity, external cues in the room, and water opacity were rigorously reproduced. Swimming was video-tracked (Viewpoint, Champagne-au-Mont-d'Or, France), with trajectories being analyzed as latencies and distances. The software divided the pool into four quadrants. Training consisted in three swims per day for 6 days, with 15 min intertrial-time interval. Start positions, set at each limit between quadrants, were randomly selected and each mouse was allowed a 90 s swim to find the platform, initially placed in the NE quadrant. If at the end of the 90 s the animal did not find the platform, it was placed and left for 20 s on it. The median latency and swim path length were calculated for each training day. An avoidance criterion was defined and animals were considered to correctly avoid staying in the water if they found the platform location within 90 s and failed to re-jump into water during 20 s. Floating (mobility lower than 1 cm/s) was also analyzed and the duration of wall hugging was measured *ex tempora* from the videotrack recordings. For swim duration, swim path length, wall hugging duration and floating duration, the median value was calculated for each training day and then averaged per day.

Twenty-four hours after the last swim on day 7, a probe test was performed during which the platform was removed and each animal allowed a free 60 s swim. The percentage of time spent in the training (T) quadrant was determined. Between days 11 and 14, mice were re-tested in the water-maze for assessing non-spatial learning. They were submitted to the acquisition of a visible platform placed in a new location, with training consisting in three swims per day with 15 min intertrial-time interval. Swimming was analyzed as latencies and distances.

Forced swimming test

Response to an acute stress was examined using a forced swimming procedure. Animals were forced to swim in a glass cylinder (Ø12 cm, height 24 cm) during 15 min on day one and during 6 min on day two. Sessions were video-tracked (ViewPoint) and quantity of movement (immobility, struggling and swimming) analyzed min per min during the 6 first minutes.

Experimental series

Animals were tested in series in the different behavioral tests assessing activity or learning and memory, finishing with the most stressful procedures: the rotarod test, open-field, Y-maze, water-maze (spatial reference memory procedure during 6 days, probe test on day 7 and visible platform procedure during 4 days) and passive avoidance tests in that order and over 21 days. Different animals were submitted to the forced swimming test and were then used for morphological studies.

Statistical analyses

Data were analyzed using one-way ANOVA (F value) or two-way ANOVA with strain and time as independent factors, followed by a Newman-Keuls' *post-hoc* test. When a cut-off time was set, for passive avoidance latencies or water-maze swimming durations, data do not follow a Gaussian distribution and non-parametric tests were used. Data were analyzed using Kruskal-Wallis ANOVA (H value) or a repeated-measures Friedman ANOVA (Q values), followed by a Dunn's *post-hoc* test. The percentages of animals-to-criterion were analyzed as 2 x 2 contingency tables and by using a Fisher's exact test. One-column comparisons, vs chance or zero levels, were performed using a t -test. The level of statistical significance in all cases was $p < 0.05$.

RESULTS

Expression of RIP140 in the central nervous system

We first examined the expression of RIP140 in the brain, particularly focusing on cortical and hippocampus areas. Since the coding sequence of RIP140 has been replaced by the LacZ sequence in the KO animals, β -galactosidase transcription is driven by the RIP140 promoter and thus reflects the expression of the endogenous RIP140 gene in wild-type (WT) mice. Using both β -galactosidase staining (Fig. 1a) and immunofluorescence (Fig. 1b), we observed a nuclear expression of RIP140 in numerous cells from the cortex and the hippocampus areas. RIP140 staining appeared widely distributed within neuronal layers throughout cortical cell layers and within pyramidal and granular cell layers of the hippocampal formation (Fig. 1b).

To examine whether RIP140 inactivation directly affected brain morphology, we performed a histological analysis of WT and RIP140 KO animals. Frontal brain sections stained with haematoxylin-eosin showed no major significant difference in the architecture, cell layer thickness and density in the cortical or hippocampal regions of the RIP140 KO mice compared to WT animals (Fig. 1c). Moreover, luxol fast blue coloration, which is commonly used for identifying the basic neuronal structure in brain or spinal cord tissue and for staining myelin as well, including phospholipids, also did not evidence grossly observable structural modifications (see Fig. 1d for a detailed magnification).

General behavioral measures in RIP140 KO mice

We then investigated whether the absence of RIP140 expression modified the general behavior of mice, by analyzing their general activity in an open-field paradigm. As summarized in Table 1a, ANOVA analyses failed to show significant variations between RIP140^{+/+}, RIP140^{+/-} and RIP140^{-/-} mice for any of the parameters measured, assessing mobility and exploration (ANOVA for locomotion: $F_{(2,40)} = 0.50$, $p > 0.05$; immobility: $F_{(2,40)} = 0.92$, $p > 0.05$; rearing: $F_{(2,40)} = 0.04$, $p > 0.05$), anxiety (departure latency: $F_{(2,40)} = 1.41$, $p > 0.05$; thigmotaxis: $F_{(2,40)} = 0.49$, $p > 0.05$), and stereotyped responses (rearing; grooming: $F_{(2,40)} = 0.25$, $p > 0.05$). The motor coordination was also assessed, using a non-accelerating rod speed procedure, on the rotarod test (Table 1b). No significant differences were found between RIP140^{+/+}, RIP140^{+/-} and RIP140^{-/-} mice (Kruskal-Wallis non-parametric ANOVA: $H_{(2)} = 1.53$ $p > 0.05$). These observations indicated that RIP140 KO animals did not show major general behavioral

deficits, as compared to WT mice, that would impede their ability to perform memory and emotional tasks.

Learning and memory impairments in RIP140 KO mice

Working memory was first assessed using the spontaneous alternation procedure, a non-aversive task based on exploratory behavior and allowing differentiation between mnesic and locomotor behaviors. The ANOVA analysis failed to detect any significant variation among strains for both the alternation percentage ($F_{(2,38)} = 0.58$, $p > 0.05$; Fig. 2a) and number of arm entries ($F_{(2,38)} = 0.93$, $p > 0.05$; Fig. 2b).

Long-term non-spatial memory was then examined using a step-down passive avoidance response. During the first training session, no difference among groups was noted in terms of step-down latency ($H_{(2)} = 1.78$, $p > 0.05$; Table 2a) or sensitivity to the shock ($F_{(2,38)} = 0.59$, $p > 0.05$; Table 2a). Moreover all groups highly significantly increased their step-down latency during the second training session (Table 2b). Two parameters were measured during the retention session: the step-down latency ($H_{(2)} = 11.48$, $p < 0.01$; Fig. 2c) and percentage of animals-to-criterion (Fig. 2d). Highly significant impairments were measured for both parameters in RIP140 KO mice as compared with heterozygous or WT animals.

Hippocampus-dependent spatial memory was assessed using place learning in the water maze. Animals were first subjected to a hidden platform procedure by learning a fixed position of platform (reference memory). The learning ability of animals was examined in terms of: (i) procedural learning, *i.e.*, the ability to understand that there is an escape to swimming and that this escape is the platform, and (ii) spatial learning. The first item could be analyzed through the percentage of animals finding the platform and remaining on it without re-jumping into the water, thus reaching the avoidance criterion, and both items contributed to swimming latencies decreasing over trials. Male RIP140^{+/+} and RIP140^{+/-} mice showed a significant decrease in swimming latencies over training trials (Friedman repeated-measure non-parametric ANOVA: $Q_{(5)} = 21.4$, $p < 0.001$ for RIP140^{+/+}; $Q_{(5)} = 17.7$, $p < 0.01$ for RIP140^{+/-}; Fig. 3a) and increase in avoidance percentage (Fig. 3b), indicating that they acquired both the procedure and platform location. On the contrary, no significant learning was measured in RIP140^{-/-} mice, in terms of swimming duration ($Q_{(5)} = 6.41$, $p > 0.05$; Fig. 3a) or avoidance percentage (Fig. 3b). Significant differences between WT and RIP140^{-/-} mice were measured for the parameters during

the last three days of acquisition (Fig. 3a, 3b). However, animals did not swim at similar speed and RIP140^{-/-} mice show a highly significant decrease in swimming speed ($F_{(2,39)} = 11.8$, $p = 0.0001$; Fig. 3c). We therefore analyzed the swim path length, as well as two parameters helping to analyze the animal's strategy in the maze: the time spent hugging the wall of the pool and the floating duration, when animals exhibit acute behavioral despair. RIP140^{+/+} and RIP140^{+/-} groups showed decreasing length to the platform among trials ($Q_{(5)} = 25.9$, $p < 0.0001$ for RIP140^{+/+}; $Q_{(5)} = 24.0$, $p < 0.001$ for RIP140^{+/-}; Fig. 3d), but not RIP140^{-/-} mice ($Q_{(5)} = 2.23$, $p > 0.05$; Fig. 3d). This measure confirmed that WT and RIP140 heterozygous groups showed a significant improvement of their ability to find the platform. The swim path length did not decrease for RIP140 homozygous mice, suggesting absence of learning. However, since the end of the session was decided by a maximum swim duration and not distance, average swim path lengths were lower for RIP140^{-/-} animals than for WT and heterozygous groups during initial training sessions.

The analysis of wall hugging showed that all groups exhibited a significant reduction of this behavior over the training days ($Q_{(5)} = 30.8$, $p < 0.0001$ for RIP140^{+/+}; $Q_{(5)} = 37.9$, $p < 0.0001$ for RIP140^{+/-}; $Q_{(5)} = 41.6$, $p < 0.0001$ for RIP140^{-/-} mice; Fig. 3e). RIP140^{-/-} animals showed higher wall hugging duration, particularly during training days 2 and 5-6 as compared with control animals (Fig. 3e). Floating duration was also analyzed. It increased significantly during training sessions ($Q_{(5)} = 29.7$, $p < 0.0001$ for RIP140^{+/+}; $Q_{(5)} = 24.6$, $p < 0.001$ for RIP140^{+/-}; $Q_{(5)} = 16.8$, $p < 0.01$ for RIP140^{-/-} mice; Fig. 3f). Moreover, the floating duration shown by RIP140^{-/-} mice was significantly higher than WT controls during training days 3-5 (Fig. 3f). However, these behavioral responses of RIP140 KO animals were both very limited during the last training sessions (7 s for wall hugging and about 5 s for floating duration) and are unlikely to account for a lack of procedural learning.

The probe test confirmed that only RIP140^{+/+} and RIP140^{+/-} animals, but not RIP140^{-/-}, learned the platform location, as they exhibited a significantly higher presence in the training (T) quadrant, as compared to the chance levels (Fig. 3g).

Given the very poor performances of RIP140^{-/-} animals, we checked their visual and swimming performances using a visible platform procedure (Fig. 3f). ANOVA analysis showed that all groups exhibited a progressive decrease in the latencies to find the platform without differences among strains ($Q_{(3)} = 18.61$, $p < 0.001$ for RIP140^{+/+}; $Q_{(3)} = 23.27$, $p < 0.001$ for RIP140^{+/-}; $Q_{(3)} = 18.15$, $p < 0.001$ for RIP140^{-/-}; Fig. 3h) and in the path length to reach the platform ($Q_{(3)} = 17.11$, $p < 0.001$ for RIP140^{+/+};

$Q_{(3)} = 21.77$, $p < 0.001$ for RIP140^{+/-}; $Q_{(3)} = 16.85$, $p < 0.001$ for RIP140^{-/-}; Fig. 3i). Collectively, these observations showed that RIP140^{-/-} mice exhibited spatial reference memory impairments in the water maze paradigm, whereas heterozygous mice presented with similar performances as compared to WT animals.

Behavioral despair in RIP140 KO mice

The response to acute stress was tested using the forced swimming stress procedure (Fig. 4). Animals were submitted to a 15-min on day 1 and 6-min session on day 2. Analysis of the total duration of immobility during the 6 first minutes of session 1 showed a significantly increased response of RIP140^{-/-} mice ($F_{(2,20)} = 7.89$, $p < 0.01$; Fig. 4a). Analysis of the immobility minute per minute using a two-way repeated-measure ANOVA revealed a significant effect of strain ($F_{(2,18)} = 8.55$, $p < 0.01$) and time ($F_{(5,90)} = 10.5$, $p < 0.0001$) but not for the interaction ($F_{(10,90)} = 0.75$, $p > 0.05$; Fig. 4b). RIP140^{-/-} animals exhibited rapidly an average immobility of 40 s per minute, while WT and heterozygous mice showed a maximum of 25 s per minute (Fig. 4b). This difference is maintained during the day 2 session when heterozygous RIP140^{+/-} mice also showed an increased immobility response. Analysis of the total duration of immobility showed significant differences ($F_{(2,20)} = 7.54$, $p < 0.01$; Fig. 4c) and analysis of the immobility minute per minute revealed a significant effect of strain ($F_{(2,18)} = 6.30$, $p < 0.01$) and time ($F_{(5,90)} = 5.02$, $p < 0.001$) but not for the interaction ($F_{(10,90)} = 0.88$, $p > 0.05$; Fig. 4c). RIP140^{-/-} animals, and at a lesser extent RIP140^{+/-} animals, showed increased duration of immobility between min 2 to min 5 (Fig. 4d). These observations clearly showed that RIP140 KO mice presented with an increased response to an acute stress as compared with WT animals.

DISCUSSION

The physiological role of RIP140 was mainly evaluated by analyzing the functional consequences of its deletion. RIP140^{-/-} mice were generated by replacing almost the entire coding region by a β -galactosidase expressing cassette (White *et al.* 2000). These mice are viable but present with strong physiological deregulations, such as female infertility and alteration of metabolism in white adipose tissue (Leonardsson *et al.* 2004) and muscle (Seth *et al.* 2007). Despite these observations on the impact of RIP140 activity on biological metabolism-related processes, nothing is known about the involvement of RIP140-mediated gene regulation in the molecular mechanisms sustaining cerebral plasticity and related human pathology.

In the mouse, RIP140 mRNA is strongly expressed in the brain (Lee *et al.* 1998) and its expression in the central nervous system is detected at the embryonic stage since 12.5 *dpc* (Reymond *et al.* 2002). Interestingly, RIP140 protein brain levels were found to be decreased in male and female mice upon aging (Ghosh & Thakur, 2008). More importantly, the gene coding for RIP140 being located on chromosome 21, an increased expression of the RIP140 has been detected in hippocampus of patients with Down's syndrome (Gardiner, 2006). In addition, its interaction with partners such as ER α and GR receptors, whose involvement in the regulation of these cerebral plasticity processes has been clearly demonstrated, strengthens the possibility of a significant implication of RIP140. Nevertheless, this important number of partners also brings a significant layer of complexity, as their respective roles are highly dependent on the context. For instance, while ER α exhibit mainly positive effects on learning and memory formation, GR can exert both positive and negative effects (see below). Altogether, although a simple predictive model of RIP140 mode of action is difficult to establish, this evidence points to an important implication of RIP140 in the regulation of cognitive functions.

Here, we investigated the phenotype of 2 month-old male RIP140 KO mice in a panel of behavioral tests. General activity responses in the open-field and performances on the rotarod showed no obvious consequences for the constitutive deletion of RIP140 gene on motor coordination and general activity behavior. Swim speed analysis in the water-maze procedure, however, clearly revealed a

lower performance for RIP140^{-/-} mice as compared with WT animals. Nevertheless, this reduction in swimming speed is likely to reflect a muscular weakness in RIP140 KO mice resulting from its role in skeletal muscle function (Seth *et al.* 2007), but is not accompanied by other alterations (ataxia, cerebellar defect) that would have lead to a severe phenotype in the rotarod.

Despite this globally normal phenotype, RIP140 KO mice exhibited marked memory impairments. First, animals showed a significant impairment in the passive avoidance response. Decreased latencies in the passive avoidance test may indicate memory deficits, reduced shock sensitivity or impaired inhibitory control. The latter two possibilities can be excluded, since data collected during the training sessions clearly indicated that shock sensitivity was similar among strains and that all strains showed a similar evolution of the step-down latency during the second training session (Table 2). Second, RIP140^{-/-} mice were not able to learn the fixed platform position in the water-maze. In this test, animals failed to reach the avoidance criterion, suggesting that they could not acquire the procedure involved in this task. However, such failure would also have been detected by an exaggerated wall hugging behavior or floating response, which both reflect either a persistence in trying to escape across the wall or a lack of perseverance and sign of behavioral despair. RIP140 KO mice showed only a moderate increase of these responses that accounted, during the last training trials, to an average of 12 s over the 90 s duration session. The lack of acquisition is therefore likely to be due to an impaired learning ability. Moreover, the visible platform procedure indicated that the visual ability of the animals was not in question, but the complexity of the task and putatively the spatial processes may be altered. Interestingly, RIP140^{-/-} animals failed to show a significant alteration of spontaneous alternation in a Y-maze, suggesting that general exploration and working memory processes remained effective. Indeed, spatial working memory deficits measured using an adequate protocol in the water-maze, such as a daily change of the platform location and training trials separated by a very short inter-trial time interval (Yamada *et al.* 1999), is systematically correlated with significant spontaneous alternation deficits (see for instance Maurice *et al.* 2008). Moreover, since the water-maze procedure relies partly on short-term memory abilities within training sessions, it suggested that the lack of behavioral flexibility shown by RIP140^{-/-} mice is related to the stressful context and aversive stimuli sustaining the acquisition processes. However, it could also be proposed that a more integrative paradigm relying on spatial performances and complex coordination between

sensorial stimuli (context composition and water) and effectors (swim and putatively spatial localization) involved in the water-maze was necessary to unveil the learning impairments. It remains, however, that RIP140^{-/-} mice showed a total lack of improvement and a very low rate of goal hits. The possibility that they failed to learn very fundamental aspects of the task, which would be needed to proceed to a stage where spatial memory becomes relevant and limiting, can not be fully excluded. In addition, RIP140^{-/-} mice suffer from physical impairments. Therefore, in order to ensure the presence of spatial memory deficits, further experiments should be considered, involving, for instance, extensive pretraining with the visible version of the task.

Noteworthy, RIP140 heterozygous mice exhibited only a trend to reduced performances in the water-maze, both in terms of acquisition profile or probe test response, but without deficit in spontaneous alternation or passive avoidance response, suggesting that the consequences of RIP140 gene deletion can only be evidenced in homozygous animals. We also addressed the efficacy of the response to an acute stress in RIP140 KO mice and observed an increased immobility in response to forced swimming stress for RIP140^{-/-} mice on day one and for both RIP140^{+/-} and RIP140^{-/-} mice on day two of the test. It should be noted that the muscular/motor deficit measured could explain part of the increased immobility response in RIP140^{-/-} mice. Therefore, similarly as for learning abilities, homozygous animals exhibited an increased alteration of the response to stress as compared with heterozygous mice.

RIP140 is strongly expressed in mouse brain and we confirmed a wide nuclear expression of the protein within neurons throughout cortical cell layers and within pyramidal and granular cell layers of the hippocampal formation (see Fig. 1). Interestingly, we did not observe significant morphological alterations in both the cortical and hippocampal formations of RIP140 KO animals, suggesting that the constitutive deletion of RIP140 gene does not lead to severe abnormalities in the development of the brain. These observations, however, do not rule out the possibility of slight developmental perturbations in the gene expression affecting cerebral plasticity integrity and leading to memory deficits at the adult stage, as we observed.

Among the nuclear receptors known to recruit RIP140, many have been involved in learning and memory process. For example, ER α and their endogenous ligands, estrogens, facilitate several forms of memory and particularly hippocampus-dependent tasks (Foy *et al.* 2008). Post-training 17 β -estradiol administration in ovariectomized female rat facilitates retention in the water-maze task (Singh *et al.* 1994). Other important partners of RIP140 are the retinoic acid receptors (RAR/RXR), which have been reported to be key proteins controlling many cerebral plasticity processes, including emotional state as well as learning and memory (O'Reilly *et al.* 2008).

Glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), have been linked to adaptation to stress and thus to the regulation of emotional state and learning and memory mechanisms (Kolber *et al.* 2008). For instance, blocking GR activation, using antisense (Korte *et al.* 1996) or antagonist (Bachmann *et al.* 2005) strategies, reduced immobility in forced swimming test. Furthermore, antidepressant treatments modulated behavioral despair through a complex regulation and fine-tuning of GR/MR expression (Heydendael & Jacobson, 2008). Several targeted mutations of GR or MR in mouse lead to severe deregulations of their learning and memory performances and corticosterone, the main glucocorticoid hormone in rodent, has been reported to affect memory processes, particularly by improving the consolidation and retrieval phases of long-term memory processes (Sandi & Rose, 1997; Coburn-Litvak *et al.* 2003). Moreover, pre-training or immediate post-training intracerebroventricular infusions of a GR, but not an MR, antagonist in rats impaired spatial memory in a water maze (Oitzl & de Kloet, 1992; Roozendaal *et al.* 1996). Additionally, post-training infusions of the GR antagonist impaired memory for an avoidance task in chicks (Sandi & Rose, 1994a,b) and blocked the enhancing effects of post-training corticosterone (Sandi & Rose, 1994a). In RIP140 KO mice, we observed water-maze deficits that were likely due to improper response to the stressful environment, rather than direct spatial reference memory impairment, and concomitant impairments in avoidance response. These deficits are coherent with impaired GR activity in RIP140 KO mice and future studies must focus on corticosteroid systems and hypothalamo-pituitary axis activity in these animals.

In summary, all the known functions of RIP140-recruiting nuclear receptors may thus explain the learning and memory deficits as well as the higher despair behavior observed in RIP140^{+/-} and

RIP140^{-/-} mice, in which all these regulatory mechanisms may be altered by RIP140 diminution or deletion. However, we recently described RIP140 as a repressor of the E2F pathway (Docquier *et al.* 2010) and it is conceivable that these transcription factors targeted by RIP140 may also be involved in the behavioral phenotype of RIP140 KO mice. The targeting of other transcription factors than nuclear receptors might explain why the knock-out for RIP140 and p/CAF genes led the same phenotype although we might have expected opposite effects. In addition, our data clearly support a benefic role of RIP140 on cognitive functions and suggest that RIP140 overexpression may attenuate the learning deficits in Down syndrome patients. This is supported by recent data obtained in mice which showed that, when transgenic animals carried a trisomic segment which encompasses the NRIP1 gene, the learning deficiency phenotype is milder (Gardiner *et al.* 2010). Indeed, while Ts65Dn mice (Reeves *et al.* 1995; Moran *et al.* 2002) or Ts1Cje mice (Sago *et al.* 1998) showed almost no improvement in the distance to platform in a hidden platform task in the water-maze, Dp(10)1Yey/+;Dp(16)1Yey/+;Dp(17)1Yey/+ mice (Yu *et al.* 2010) showed a significant learning ability, that however remained significantly lower than control littermates. The latter model carried trisomic segments covering the three Hsa21 syntenic regions from mouse chromosomes 10, 16 and 17, *i.e.*, including a third copy of the NRIP1 gene. Our data would therefore support the idea that a supplemental copy of RIP140 could directly attenuate the intensity of the observed deficits.

RIP140 exerts its repressive activity in part by recruiting enzymatic activity such as HDAC at the close vicinity of the promoter, thereby inducing chromatin remodeling towards a more compacted state associated with transcriptional repression (Wei *et al.* 2000). Histone modifying enzymes, particularly histone acetyltransferase and HDAC, have been extensively associated with the molecular mechanisms underlying learning and memory processes. Histone acetylation has thus been observed at memory-enhancing gene promoters (Miller & Sweatt 2007). Interestingly, histone deacetylation and repression of transcription at memory-suppressor gene promoter has also been identified as necessary for learning and memory, especially for long-term memory storage. Moreover, the functional nature of these modifications is dependent on the gene and promoter context (Miller *et al.* 2008). Therefore, a correct learning procedure requires a complex succession of epigenetic mechanisms relying on a fine-tuning of histone-modifying enzymes. Perturbations of this very complex system, by

decreasing or removing RIP140 and its HDAC recruitment, is then likely to produce strong alterations of transcriptional regulation that might induce, at the behavioral level, strong cognitive impairments.

In conclusion, we report here for the first time a link between the transcriptional coregulator RIP140 and the neurophysiological mechanisms underlying memory formation and response to stress. Further work will be needed to precisely define its specific targets mediating this effect and its biological relevance in human neuropathology.

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LEGENDS FOR THE FIGURES

Figure 1. Brain histology in WT and RIP140 KO mice. β -galactosidase staining (a) and RIP140 immunofluorescence (b) demonstrated that RIP140 is expressed in the cortical and hippocampal regions. Haematoxylin-eosin (c) and luxol fast blue (d) staining showed no major differences in brain morphology of RIP140 KO mice compared to WT animals. Scale bars: 500 μ m.

Figure 2. Performances of RIP140 KO mice in behavioral procedures assessing working memory (a, b) and long-term memory (c, d). Spontaneous alternation test in the Y-maze: (a) percentage of alternation and (b) total number of arm entries, recorded during the 8-min session. Passive avoidance test: step-down latency (c) and (d) percentage of animals-to-criterion during the retention session. $n = 11-18$ per group. ** $p < 0.01$ vs. +/+ data, Dunn's test in (c), Fisher's exact test in (d).

Figure 3. Performances of RIP140 KO mice in the water-maze test. Mice were submitted to acquisition of the location of an invisible platform, placed in the NE quadrant of the pool, during 6 days (3 swims per day). The swim duration to find the platform (up to 90 s) was recorded (a), as well as the percentage of animals avoiding swimming, *i.e.*, finding the platform within 90 s (b), the swimming speed (c), and the swim path length (d). Wall hugging duration (e) and floating duration (f) were also analyzed. $n = 11-17$ per group. * $p < 0.05$, ** $p < 0.01$ vs. +/+ data; Dunn's test in (a, g) and Fisher's exact test in (b). On day 7, the platform was removed and mice were submitted to a probe test (g). The presence in the training quadrant was analyzed vs. chance level (25%), # $p < 0.05$, one-column *t*-test. Finally, the platform was moved to the NW quadrant and rendered visible by placing a flag onto it. Acquisition was tested during 4 days, with 3 swims per days, 15 min ITI and analyzed in terms of swim duration (f) and swim path length (i).

Figure 4. Performances of RIP140 KO mice in the forced swimming test: day 1 session (a, b) and day 2 session (c, d). (a, c) total immobility duration during the 6 first min of the session and (b, d) immobility duration analyzed minute per minute. $n = 5-9$ per group. * $p < 0.01$, ** $p < 0.01$ vs. +/+ data; Newmann-Keuls' test.

Table 1. Behavioral parameters measured in the open-field and rotarod test.

Parameter	+/+	+/-	-/-
Weight (g)	22.1 ± 0.8	20.5 ± 0.7	29.2 ± 0.5
n	12	18	11
<i>(a) Open-field test</i>			
Latency to start (s)	0.9 ± 0.5	0.2 ± 0.2	0.2 ± 0.2
Locomotion (m)	31.8 ± 2.0	30.8 ± 1.9	33.9 ± 2.7
Loc. in center (%)	28.3 ± 2.5	31.2 ± 1.9	30.4 ± 1.9
Speed (m/min)	3.38 ± 0.19	3.28 ± 0.17	3.54 ± 0.28
Immobility (s)	34.6 ± 7.3	39.3 ± 9.6	22.4 ± 4.3
Rearing	21.8 ± 2.2	22.6 ± 3.2	21.3 ± 4.6
Grooming	3.6 ± 0.5	4.1 ± 0.3	4.0 ± 0.9
Defecations	2.0 ± 0.5	2.1 ± 0.4	1.2 ± 0.4
<i>(b) Rotarod test</i>			
Falling latency (s)	378 [296-600]	600 [370-600]	452 [306-600]

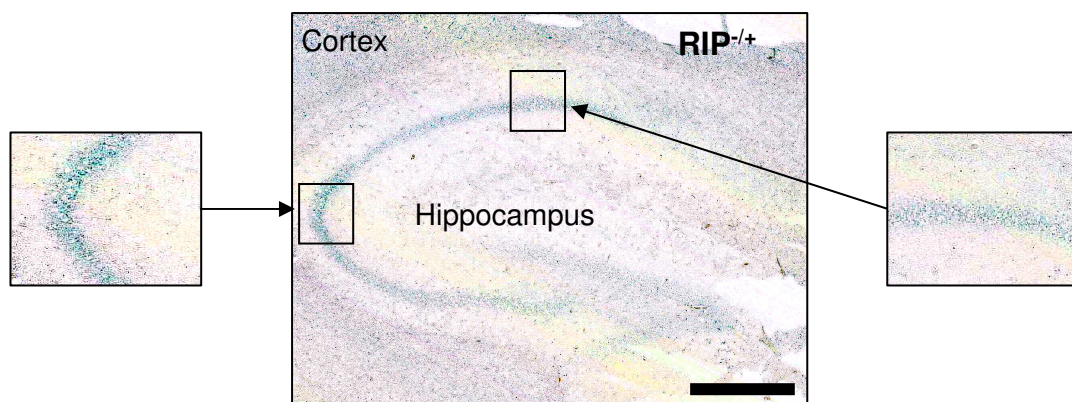
Note that falling latencies are presented as median and interquartile [25%-75%] ranges.

Table 2. Behavioral parameters measured during passive avoidance training.

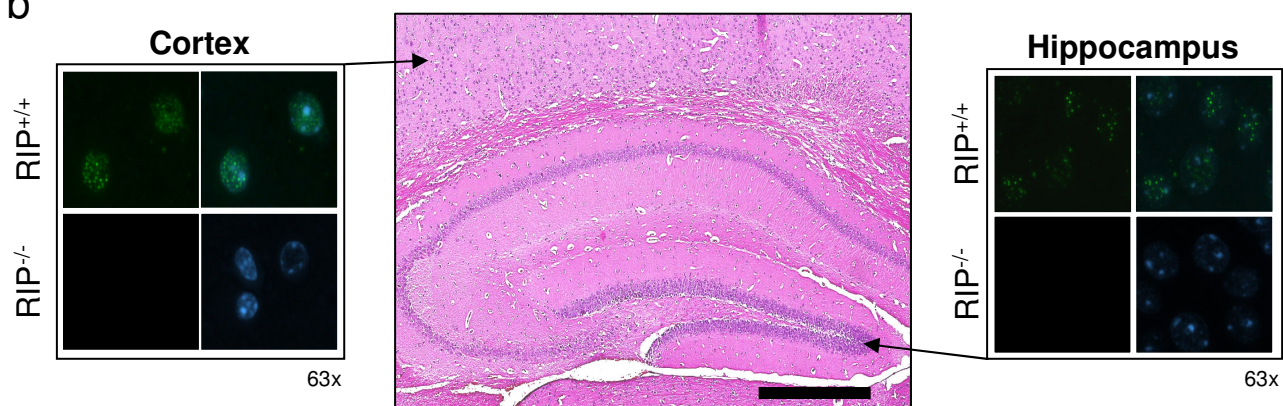
Parameter	+/+	+/-	-/-
n	12	18	11
<i>(a) First training session</i>			
Step-down latency (s)	3 [3-6]	3 [3-4]	5 [3-7]
Shock sensitivity	22 \pm 2	19 \pm 3	17 \pm 3
<i>(a) Second training session</i>			
Step-down latency (s)	49 [20-60] **	16 [3-60] **	45 [5-60] **

Note that step-down latencies are presented as median and interquartile [25%-75%] ranges. ** $p < 0.01$ vs. latency measured for the same group during the first training session (Wilcoxon matched pairs test).

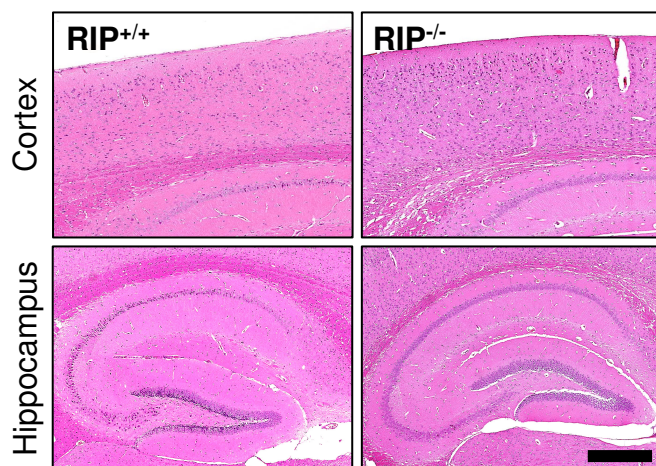
a



b



c



d

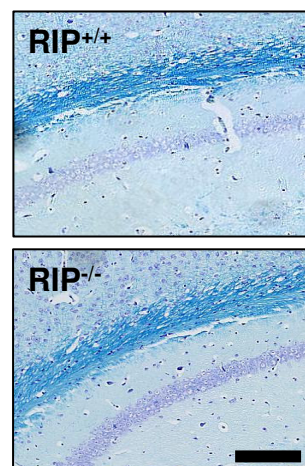


Figure 1

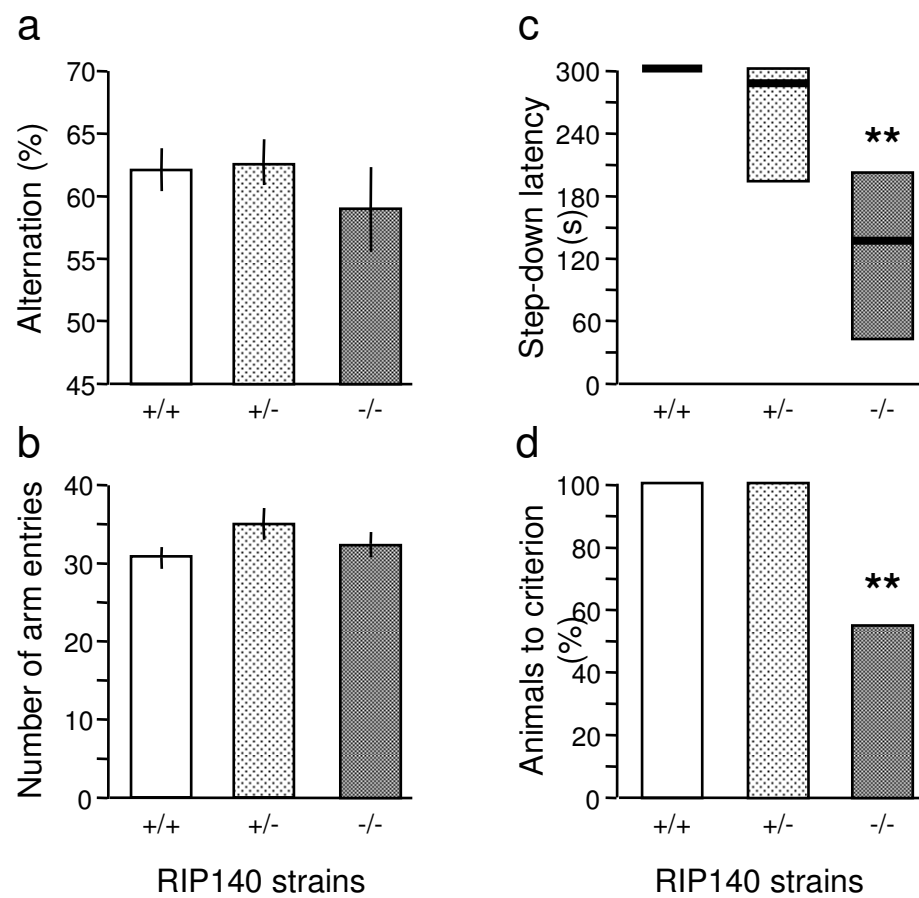


Figure 2

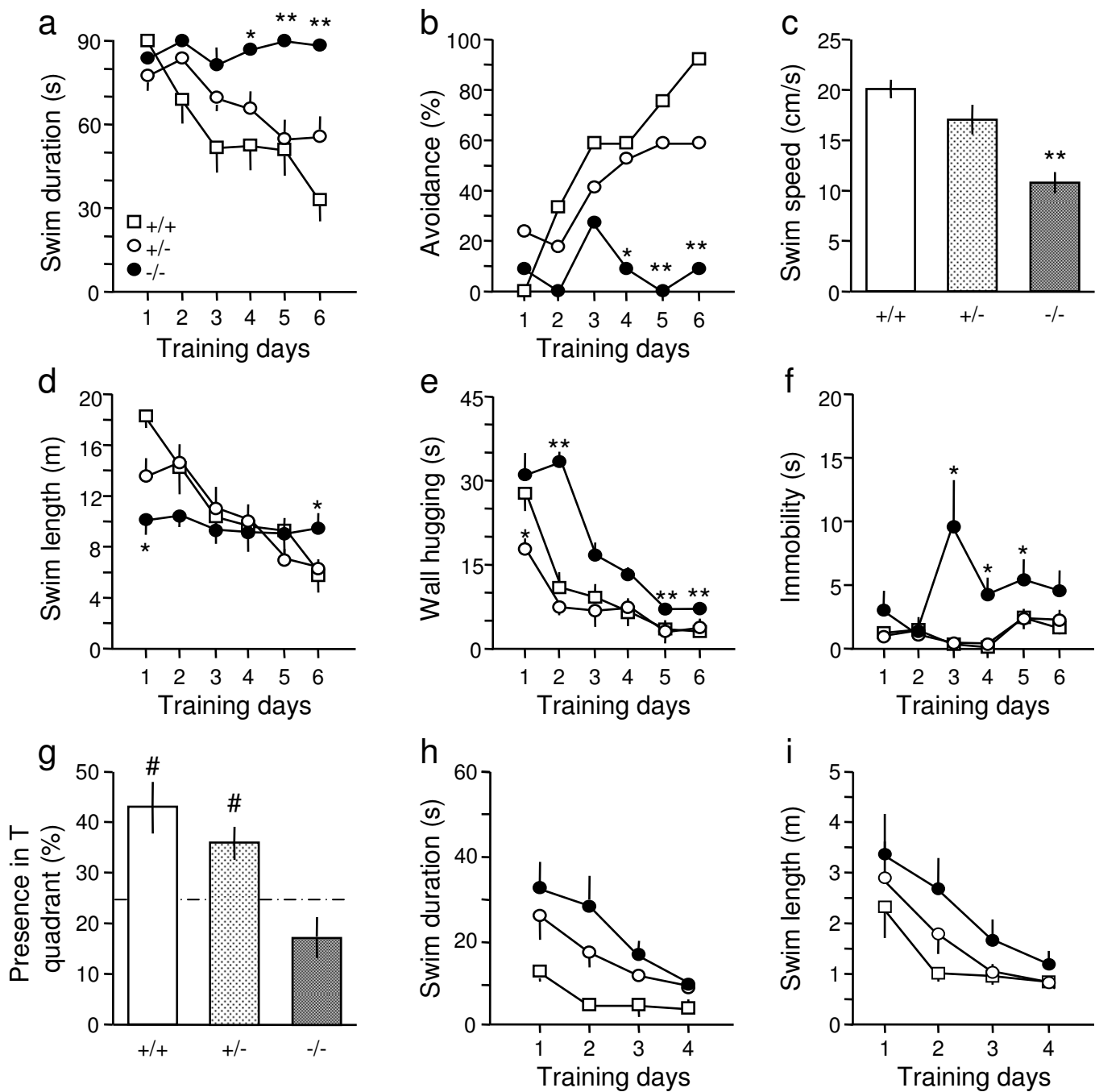


Figure 3

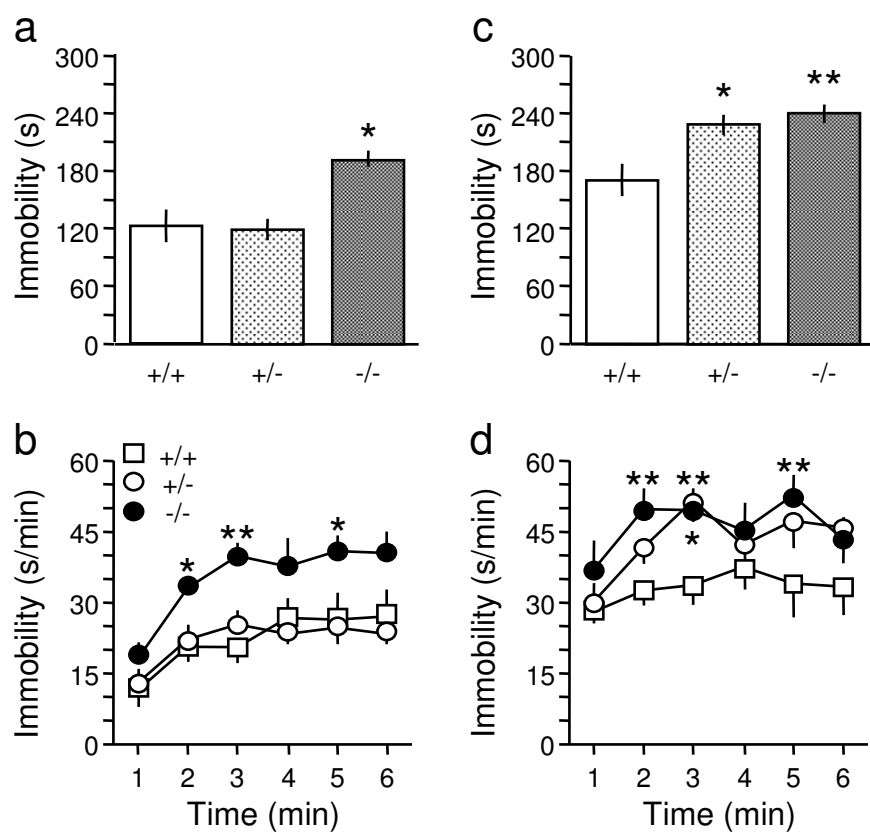


Figure 4