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Behavioural phenotyping, learning and memory in young and aged growth hormone-releasing hormone-knockout mice

Sheila Leone^{1,*}, Lucia Recinella^{1,*}, Annalisa Chiavaroli¹, Claudio Ferrante¹, Giustino Orlando¹, Michele Vacca¹, Roberto Salvatori² and Luigi Brunetti¹

¹Department of Pharmacy, G. d'Annunzio University, Chieti, Italy

²Division of Endocrinology, Diabetes and Metabolism, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Correspondence should be addressed to G Orlando: giustino.orlando@unich.it

*(S Leone and L Recinella contributed equally to this work)

Abstract

Background: Growth hormone-releasing hormone (GHRH) plays an important role in brain functions. The aim of this study was to examine cognitive functions and emotional behaviour in a mouse model of isolated GH deficiency due to bi-allelic ablation of the GHRH gene (GHRH knockout, GHRHKO).

Methods: Learning, memory and emotional behaviour were evaluated using a series of validated tests (Morris water maze, eight-arm radial maze, open field, elevated plus maze test, forced swim tests) in 2-, 5- and 12-month-old male mice either homozygous (−/−) or heterozygous (+/−) for the GHRHKO allele.

Results: Compared with age-matched +/− mice, −/− mice showed decreased cognitive performance in Morris water maze and eight-arm radial maze tests. By comparing the effects of aging in each genotype, we observed an age-related impairment in test results in +/− mice, while in −/− mice a significant decline in cognitive function was found only in 12 months compared with 2-month-old mice, but no difference was found between 5 months old vs 2 months old. −/− mice showed increased exploration activity compared to age-matched +/− controls, while both strains of mice had an age-related decrease in exploration activity. When evaluated through open field, elevated plus maze and forced swim tests, −/− mice demonstrated a decrease in anxiety and depression-related behaviour compared to age-matched +/− controls.

Conclusions: Our results suggest that homozygous ablation of GHRH gene is associated with decreased performance in learning and memory tests, possibly linked to increased spontaneous locomotor activity. In addition, we observed an age-related decline in cognitive functions in both genotypes.

Key Words

- ▶ growth hormone-releasing hormone knockout
- ▶ learning
- ▶ memory
- ▶ locomotor activity
- ▶ anxiety
- ▶ depression

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Introduction

Growth hormone (GH)-releasing hormone (GHRH), GH and insulin-like growth factor-1 (IGF-1), in addition to their recognised metabolic and endocrine effects, play a pivotal role in brain functions (1, 2). Serum GH and IGF-1 levels decrease with aging, and the possibility

that the GHRH/GH/IGF-1 axis is involved in age-related cognitive deficits is suggested by several studies (2, 3, 4). Accordingly, in rodents, the age-related decline in spatial and reference memory is reversed by the administration of GH or IGF-1 (3, 4). Additionally, GH and IGF-1 are able to

attenuate the age-related changes in hippocampal short-term plasticity and spatial learning (3, 5). Furthermore, IGF-1 deficiency in rats leads to reduction in brain microvascular density, resulting in decreased cerebral blood flow (4). Recent studies have demonstrated an important role of the IGF-1 and insulin-related signalling pathways in the control of longevity in mice (6). Indeed, GH-deficient *Ames* dwarf mice, GH receptor/GH binding protein-knockout (GHRKO) GH-resistant mice and GHRH-knockout (GHRHKO) mice show increased lifespan (7, 8). Both *Ames* and GHRKO strains do not show altered behavioural, learning and memory parameters, but lack the decline in cognitive ability in the Morris water maze test that is observed in WT mice (9, 10). GHRHKO mice represent an animal model of isolated GH deficiency with otherwise normal pituitary function (11). We have recently demonstrated that the generalised ablation of the GHRH gene is associated with anxiolytic and antidepressant behaviour, as well as hyperactivity in young mice (12, 13). This fits with the observation that in WT mice the GHRH antagonist MZ-4-71 induces antidepressant-like effects in a modified forced swim test, has anxiolytic effects in elevated plus maze test and improves memory consolidation in passive avoidance learning, with no effects on ambulation in open field test (14, 15). The aim of the present study was to further investigate the effects of aging on cognitive function and emotional behaviour in GHRHKO mice.

Materials and methods

Animals and ethics

Homozygous mice carrying a targeted ablation of the GHRH gene were previously described (11). We used 32 male animals homozygous for GHRHKO allele (−/−) and 32 normal size male mice heterozygous for the GHRHKO allele (+/−) from the same genetic background as control group, allowing comparison of animals with identical genetic background. We used 2-, 5- and 12-month-old animals (6–8 animals for each age group and genotype). GHRHKO offspring was generated by mating heterozygous males and females, as previously reported (11). Only male mice were used to avoid any possible involvement of periodical hormonal changes in female mice. The animals were housed in plexiglass cages (2–3 animals per cage; 55 × 33 × 19 cm) and maintained under standard laboratory conditions (21 ± 2°C; 55 ± 5% humidity) on a 14/10-h light/darkness cycle, with *ad libitum* access to water and food (16). They were fed a standard rodent chow

(Prolab RMH2500, PMI Nutrition International, Brentwood, MO, USA). Housing conditions and experimentation procedures were strictly in agreement with the European Community ethical regulations (EU Directive n. 63/2010) on the care of animals for scientific research. In agreement with the recognised principles of 'Replacement, Refinement and Reduction of Animals in Research', we used control group animals randomised in our previous experiments approved Italian Health Ministry (Project n. 955/2016-PR).

Morris water maze test

Learning and memory were tested with Morris water-maze test (17, 18). The apparatus consists of a circular white pool (80 cm in diameter and 55 cm in height) filled to a depth of 15 cm with water (27 ± 1°C) rendered opaque with milk powder (19). A circular platform (7 cm in diameter) was placed in one of the pool quadrants and submerged 1 cm below the water surface, so that it was invisible at water level. The first experiment day was dedicated to swimming training for 60s in the absence of the platform. During the five subsequent days, the animals were given four trial sessions per day with the platform in place. During each trial session, the time taken to find the hidden platform (latency) was recorded. Animals were subjected to a 5-day training sequence (to assay learning) then, 2 days after the end of learning assay, to a 1-day training (to assay memory).

Eight-arm radial maze test

Working and reference memory were tested with an eight-arm radial maze (20). The apparatus included a central platform (30 cm in diameter) with eight-arms (70 × 10 cm) surrounded by walls (10 cm in height) and placed in a sound and light isolated room (2 Biological Instruments, Besozzo VA, Italy). During training, animals were single housed and maintained at 80–90% of their body weight by dietary restriction. To make animals acquainted with radial maze, mice received one daily habituation session for 4 days prior to training. During the habituation trial, all the eight arms were baited with 1 food pellet, and the mouse was allowed to explore freely until it had taken all the pellets. After habituation, all mice were tested with 3 trials per day for 8 days. During the test trial, four randomly selected arms were baited with 1 pellet of food each; the baited arms were not changed throughout the experiment. The mouse was allowed to move freely in the maze until it collected the four pellets of food or until 10 min had elapsed, whichever occurred first. Entry into

a non-baited arm was scored as a reference memory error, while re-entry into baited arms that had been previously visited was scored as a working memory error. The decrease in the number of arm entries per trial was used to indicate memory acquisition.

Locomotor activity/open field test

Locomotor activity was recorded in the home cage over 10 min by a video camera (SSC-DC378P, Biosite, Stockholm, Sweden) positioned in the top-centre of a cage (35×20×13 cm) and connected to a computer. Separate video frames were acquired with a highly accurate, programmable, monochrome frame grabber board (Data Translation™, type DT3153). The apparatuses were purchased from 2 Biological Instruments (Besozzo VA, Italy). The intelligent software Smart version 2.5 (Panlab, sl Bioresearch and Technology, Barcelona, Spain) was used for data processing, automatically recording total distance travelled (cm), number of Z-beam and duration of stereotypic behaviour (sec) (self-grooming and scratching), for 10 min (12, 13, 21). We found no significant differences in locomotor activity between WT and heterozygous animals (data not shown).

To evaluate anxiety-like behaviour, each animal was placed in an open field Plexiglas box (40×40×31 cm) with a white laminated sheet of paper marked into twenty five squares (8×8 cm each) covering the floor. Each animal was monitored for 10 min. In the open field test, the distance travelled and time spent into the centre of observation chamber were recorded (12).

At the end of test, the animal was returned to its home cage, and the apparatus was cleaned with 75% ethanol and dried.

Elevated plus maze test

The apparatus consisted of two open arms and two closed arms that extended from a common central platform, elevated to a height of 45 cm above floor level and mice were individually placed in the centre of the maze facing an open arm (12). The time spent on open arms and the number of transitions between the arms were recorded during a 10-min test period.

Forced swim test

The forced swim test was based on the original version of the Porsolt forced swim test for mice (22) with modifications. Mice were forced to swim individually in a glass cylinder,

filled with water to a height of 20 cm. The temperature of the water was adjusted to $28\pm 1^\circ\text{C}$ to avoid hypothermia (23). The total time that mice spent remaining immobile was measured. Immobility was determined when the mouse was only making movements necessary to keep its head above the water and maintained a stationary posture. In this posture, forelimbs of the mouse are motionless and directed forward, the tail is directed outward and the hind legs are in limited motion. No animals showed difficulty in swimming or in staying afloat. Mice were placed in water to swim for a single trial of 15 min, and immobility was recorded during the last 4 min of the trial. Water in the cylinder was changed for each mouse (12).

Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 5.01 for Windows (GraphPad Software). All data were expressed as means±s.e.m. and analysed by two-way ANOVA followed by Bonferroni *post hoc* test. Statistical significance was accepted at $P<0.05$. Finally, as regards to the animals randomised for each experimental group, the number was calculated on the basis of the 'Resource Equation' (24), according to the guidelines suggested by the 'National Centre for the Replacement, Refinement and Reduction of Animals in Research' (NC3RS) and reported on the following web site: <https://www.nc3rs.org.uk/experimental-designstatistics>.

Results

Morris water maze test

Compared with age-matched +/- mice, -/- mice showed increased mean time to find hidden platform both during the first (assay of learning, Fig. 1) ($F_{11/9}=7.866$, $P=0.0045$) and the second training session (assay of memory, Fig. 2) ($F_{6/6}=18.76$, $P=0.0024$) in all 3 age groups, consistent with a decreased cognitive performance in GHRH-deficient animals. By comparing the effects of aging in each genotype, we observed an age-related impairment in test results in +/- mice at 5 and 12 months, while in -/- mice a significant decline in cognitive function was found only at 12 months compared with 2-month-old mice. Moreover, our findings showed a decreased percentage age-related cognitive decline in -/- mice as compared to +/- controls, during both the assay of learning (cognitive decline % (means±s.e.m.): Day 1: +/-, 5.5 ± 2.0 ; -/-, 5.8 ± 2.0 ; Day 2: +/-, 50.4 ± 0.3 ; -/-, 25 ± 1.0 ; Day 3: +/-, 53.5 ± 0.3 ; -/-, 30 ± 0.4 ; Day 4: +/-, 67.6 ± 0.2 ; -/-,

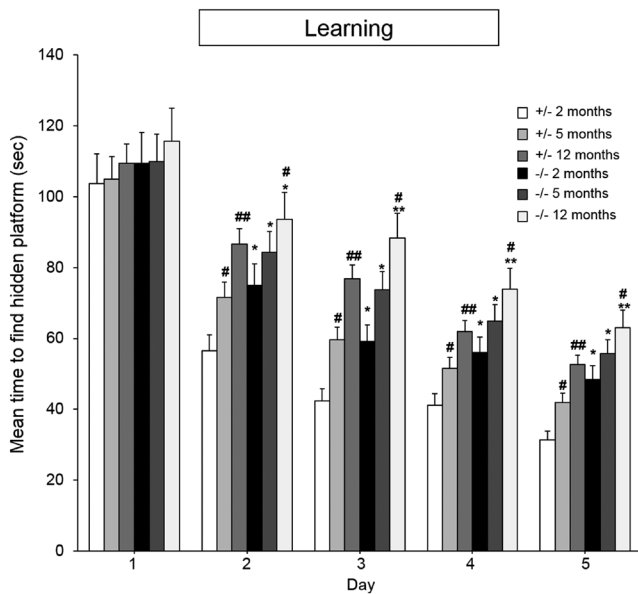


Figure 1 Morris water maze test in 2-, 5- and 12-month-old mice. Time to find hidden platform in the water maze during the first training session (assay of learning). Data are expressed as means \pm S.E.M. * $P < 0.05$; ** $P < 0.005$ vs age-matched +/- mice; # $P < 0.05$, ## $P < 0.005$ vs genotype-matched 2-month-old mice.

31.9 \pm 1.0; Day 5: +/-, 81.0 \pm 3.0; -/-, 49.0 \pm 2.0; $P = 0.004$) and memory (cognitive decline % (means \pm S.E.M.): +/-, 91.9 \pm 1.0; -/-, 31.4 \pm 1.0; $P < 0.001$).

Eight-arm radial maze test

The total number of arm entries required to collect the food pellets or reach the cut-off time was significantly higher in -/- mice compared to age-matched +/- controls

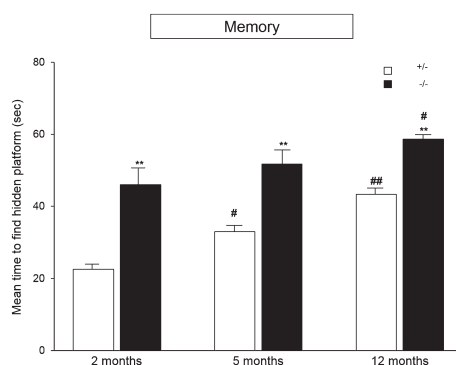


Figure 2 Morris water maze test in 2-, 5- and 12-month-old mice. Time to find hidden platform in the water maze during the second training session (assay of memory). Data are expressed as means \pm S.E.M. * $P < 0.05$; ** $P < 0.005$ vs age-matched control; # $P < 0.05$, ## $P < 0.005$ vs genotype-matched 2-month-old mice.

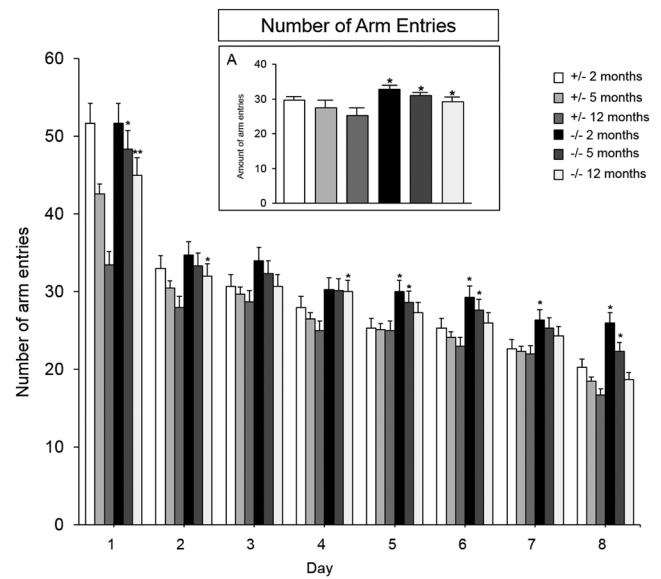


Figure 3 Eight-arm radial maze test in 2-, 5- and 12-month-old mice. Each value represents the number of arm entries made until the mouse acquired all the rewards. Data are expressed as means \pm S.E.M. on each of eight training days. Graph insert shows the average of all trial days. * $P < 0.05$; ** $P < 0.005$ vs age-matched +/- mice.

(Fig. 3) ($F_{3/18} = 3.16$, $P = 0.0043$). Furthermore, the number of working (Fig. 4) and reference (Fig. 5) memory errors was significantly higher in -/- mice compared to age-matched +/- controls ($F_{3/18} = 3.16$, $P = 0.0024$; $F_{3/18} = 3.16$, $P = 0.0092$). While an age-related memory decline was consistently seen throughout all age groups in +/- mice, in -/- mice, it was observed only when comparing 12-month vs 2-month-old animals. In addition, we found a decreased percentage age-related cognitive decline in -/- mice as compared to +/- controls, during the working (cognitive decline % (means \pm S.E.M.): +/-, 24.01 \pm 2.0; -/-, 9.05 \pm 3.0; $P < 0.001$) and reference memory test (cognitive decline % (means \pm S.E.M.): +/-, 34.25 \pm 3.0; -/-, 13.28 \pm 2.0; $P < 0.001$).

Exploration behaviour analysis

Locomotor activity recordings show that 2-, 5- and 12-month-old -/- mice display increased travelled distance and number of Z-beam breaks compared to their age-matched +/- controls (Fig. 6A and B) ($F_{2/9} = 4.26$, $P = 0.0011$; $F_{2/23} = 4.23$; $P = 0.0048$, respectively). The age-related decrease in locomotor activity is consistent in both genotypes. In addition, two-way ANOVA showed that -/- mice displayed a significant decrease of the stereotypic behaviour respect to +/- mice (self-grooming/scratching (s)

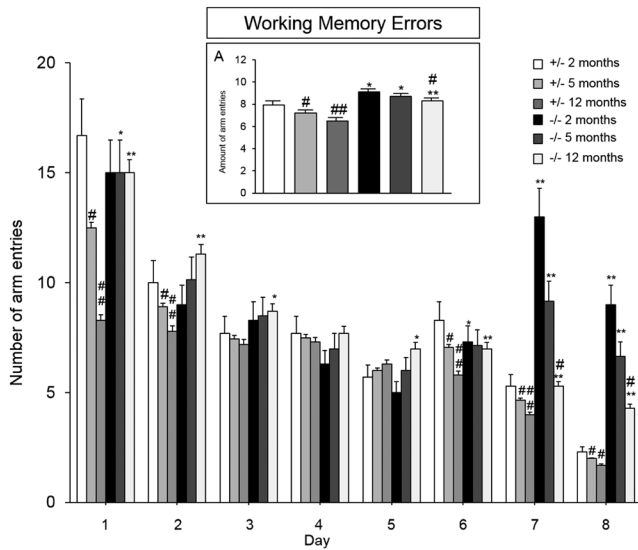


Figure 4 Eight-arm radial maze test in 2-, 5- and 12-month-old mice. Each value represents number of working memory errors made until the mouse acquired all the rewards. Data are expressed as means \pm S.E.M. on each of eight training days. Graph insert shows the average of all trial days. * $P < 0.05$; ** $P < 0.005$ vs age-matched +/- mice; # $P < 0.05$, ## $P < 0.005$ vs 2-month-old genotype-matched mice.

(means \pm S.E.M.): +/- 2 months, 76.17 ± 0.48 ; -/- 2 months, 31.67 ± 0.49 ; +/- 5 months, 75.01 ± 0.12 ; -/- 5 months, 32.30 ± 0.50 ; +/- 12 months, 78.33 ± 0.95 ; -/- 12 months, 33.33 ± 0.88 ($P < 0.05$).

Anxiety-like behaviour

In the open field test, 2-, 5- and 12-month-old -/- mice travelled longer distance and spent significantly more time in the central zone compared to age-matched controls (Fig. 7A and B) ($F_{2/19} = 3.56$, $P = 0.0034$; $F_{2/19} = 3.56$, $P = 0.0034$; respectively). Similarly, in elevated plus maze test, -/- mice demonstrated a decrease in anxiety-related behaviour (Fig. 7C, D and E), compared to age-matched controls. 2-, 5- and 12-month-old -/- mice spent more time in open arms and increased transitions compared to age-matched controls ($F_{2/21} = 3.47$, $P = 0.036$). An age-related decline in locomotor activity was seen in both +/- and -/- mice.

Behavioural despair

Figure 8 shows the total immobility time in the forced swim test. 2-, 5- and 12-month-old -/- mice showed a decrease in immobility time compared to age-matched controls. An age-related decline in immobility time was seen in both +/- and -/- mice ($F_{2/23} = 5.66$, $P = 0.0001$).

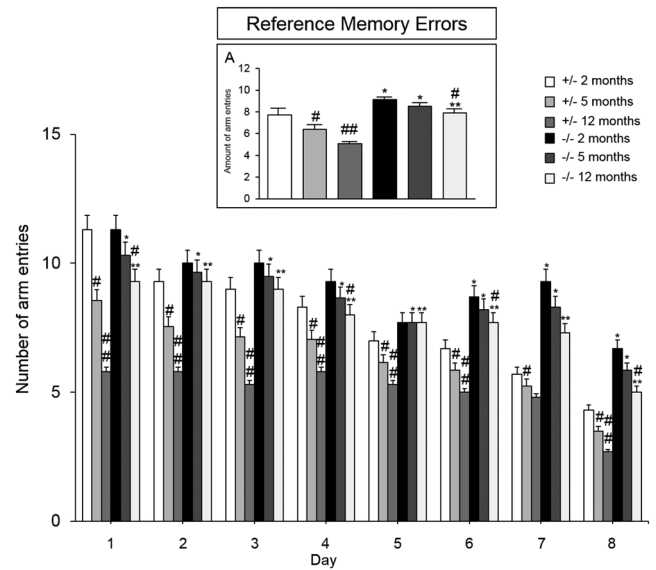


Figure 5 Eight-arm radial maze test in 2-, 5- and 12-month-old mice. Each value represents number of reference memory errors made until the mouse acquired all the rewards. Data are expressed as means \pm S.E.M. on each of eight training days. Graph insert shows the average of all trial days. * $P < 0.05$; ** $P < 0.005$ vs age-matched +/- mice; # $P < 0.05$, ## $P < 0.005$ vs 2-month-old genotype-matched mice.

Discussion

The GHRH-GH-IGF-1 axis is known to play a key role in cognition and behaviour. Kinney and coworkers (9) showed that old GH-resistant mice had better retention of footshock inhibition learning compared to their age-matched normal siblings. A beneficial role of decreased GH action on learning in mice has been hypothesised by Basu and coworkers (25). The same authors reported that transgenic mice secreting excess bovine GH showed significantly decreased spatial learning and memory, while

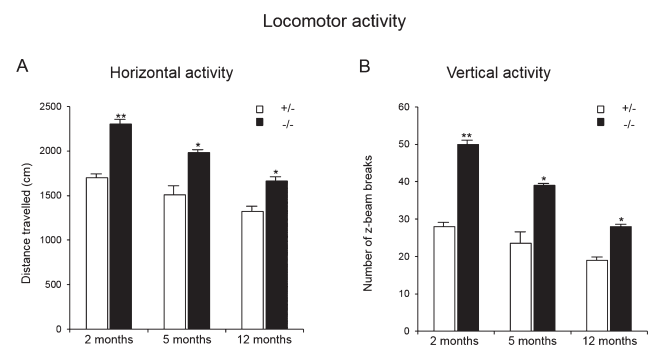


Figure 6 Locomotor activity in 2-, 5- and 12-month-old mice. Exploration activity was measured as horizontal activity (A) and vertical activity (B). Data are expressed as means \pm S.E.M. * $P < 0.05$; ** $P < 0.005$ vs age-matched +/- mice.

A Open Field

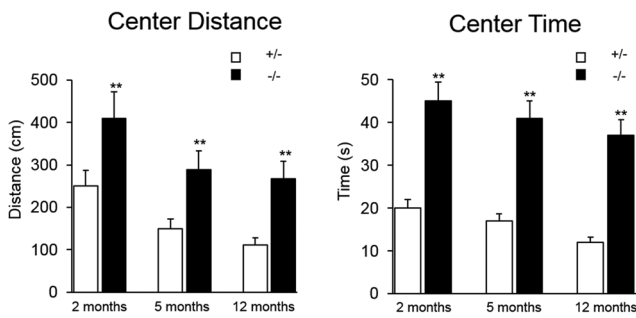


Figure 7
Analysis of anxiety-related behaviour in 2-, 5- and 12-month-old mice. The animal mice display decreased levels of anxiety-like behaviours. (A) Open field, (B) elevated plus maze. Data are expressed as means ± s.e.m. * $P < 0.05$; ** $P < 0.005$ vs age-matched +/- mice.

transgenic mice expressing GHR antagonist showed better learning parameters than control animals, suggesting a detrimental effect of GH on spatial learning and memory (25). Interestingly, it has been hypothesised that GH receptor deficiency or primary pituitary disorders in mice could have a cognitive benefit from these deficits (10). Conversely, our results, obtained using a multitasking approach, show that GH deficiency due to ablation of the GHRH gene has a negative effect on learning and memory performance. Using the hidden platform in Morris water maze test, we found that -/- mice showed decreased learning (first training session, Fig. 1) and memory performance (second training session, Fig. 2) when compared with age-matched control animals. Similar results are shown in eight arm radial maze test, with a significant impairment in working and reference memory in -/- mice (Figs 3, 4 and 5).

In a previous study, we have shown that young GHRHKO mice present increased locomotor activity, possibly related to reduced NE levels in ventral striatum (13). The present data confirm the increased spontaneous activity in young -/- mice, and expand the same

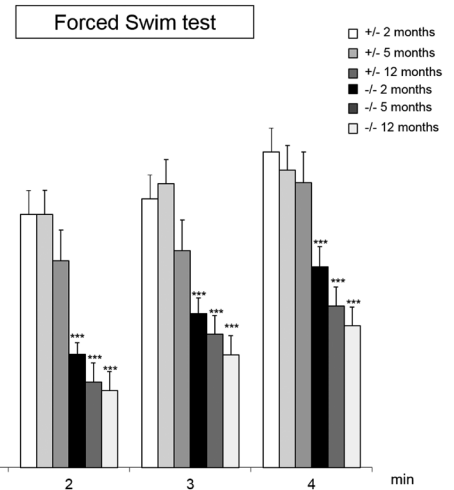


Figure 8
Behavioural despair measured in the forced swim test in 2-, 5- and 12-month-old mice. The -/- mice display decreased levels of despair behaviour. Data are expressed as means ± s.e.m. *** $P < 0.0001$ vs age-matched +/- mice.

results also to aged animals, showing that GHRH gene homozygous deletion leads to increased locomotion in all age groups. Several studies observed a decrease in locomotor activity with aging in mice (9, 26). Kinney and coworkers (10) found that GH-resistant GHRKO mice did not experience this age-related decline. Similar observation was made in Ames dwarf mice (10). By contrast, we found an age-related reduction in total distance travelled in both -/- and +/- mice (Fig. 6). Since high levels of vigorous activity may interfere with learning and memory performance tests (27, 28), we speculate that lower cognitive performance in -/- animals is consequential to their increased locomotion.

Our experiments also focused on the effects of GHRH gene ablation on age-related cognitive impairment. Our results are consistent with a decrease in cognitive functions, as evaluated through Morris water maze and eight arm radial maze tests, both in GHRHKO and control mice. However, while in control mice, the cognitive decline is consistent through all age groups, in -/- mice a significant impairment in test results is observed only at 12 months (Figs 4, 5 and 6).

The role of the GHRH-GH-IGF-1 axis in aging is still a matter of controversy. There is accumulating evidence that in mice impairment of the somatotrophic axis is strongly related to beneficial effects on longevity, with anti-aging and life-extending actions (6). Several mouse models of GH deficiency or GH resistance, including Ames dwarf, GHRKO and GHRHKO (the strain we used in this work) mice have increased lifespan (7, 8, 29).

Recently, levels of GH and IGF-1 during early life have been suggested to be critical in determining susceptibility to cancer later in life (30). In this context, a decrease in extended lifespan and increase in carcinogenesis was found in *Ames* dwarf mice, exposed to GH early in life (31). Thus, the effects of a suppressed but consistent GH action may very well have a longevity phenotype distinct from the other two models.

Actually, it is well established that aged animals have reduced performances in learning and memory tasks (32, 33, 34, 35). Indeed, several studies suggest that the administration of GH and IGF-1 is able to reverse the age-related decline in spatial and reference memory (2, 3), as well as attenuate age-related variations in hippocampal short-term plasticity and spatial learning in non-GH-deficient animals (3, 4, 5). On the other hand, animal models of GH axis deficiency have not consistently reported an age-related impairment of cognitive performance. Both old *Ames* dwarf and GHRKO mice did not show a significant difference in cognitive ability and performance in behavioural and learning and memory parameters with respect to young controls (9, 10, 36), suggesting that GH axis-deficient animals are protected from age-related memory decline. Interestingly, Sun and colleagues (8) reported that *Ames* dwarf mice have normal GH and IGF-1 mRNA levels in the hippocampus. This may explain the normal central nervous system functioning independently from pituitary GH secretion. We have also observed that $-/-$ mice have decreased anxiety- and depression-related behaviour (Figs 7 and 8), excluding any stress-induced behavioural inhibition, confirming previous results (12). Additionally, $-/-$ mice demonstrated a reduction of the stereotypic behaviour, such as self-grooming and scratching, which are considered a useful index of anxiety behaviour, in animal models (37). Interestingly, we observed an age-related decline in anxiety- and depression-related behaviour in both $-/-$ and control mice (Figs 7 and 8). The combined effects of aging and GH deficiency on anxiety and depression have not been previously investigated yet. Mood dysfunctions have been reported to be specifically related to GH deficiency (38, 39), but the anxiolytic-antidepressant effects of GHRH antagonist suggests that GHRH itself may be involved in behavioural control (14, 15).

In conclusion, our results suggest that homozygous ablation of the GHRH gene is associated with decreased performance in learning and memory tests, possibly linked to increased spontaneous locomotor activity. In addition, we observed an age-related decline in cognitive functions in both genotypes. Future studies will investigate whether

cognitive impairment is a consequence of GH deficiency or neuronal GHRH knockout, with possible accompanying CNS neurotransmitter alterations.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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