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Behavioural phenotyping of male growth hormone-releasing hormone (GHRH) knockout mice

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ABSTRACT

Objective: GH-releasing hormone (GHRH) is a key regulator of GH secretion. The role of GH in anxiety is somewhat contradictory. The aim of this study is to elucidate the consequences of lack of GHRH on emotional behaviour in a mouse model of GH deficiency due to removal of the GHRH gene (GHRH knock out, GHRHKO).
Design: Homozygous GHRHKO and wild type male mice were utilized for this study. The emotional behaviour was measured through a battery of behavioural tests (locomotor activity/open field, light–dark exploration, elevated plus maze, forced swim test, tail suspension test). To correlate the emotional behaviour with brain neurochemistry, we evaluated thyrotropin-releasing hormone (TRH) gene expression in hypothalamic tissue by real-time PCR, and the levels of norepinephrine (NE), dopamine (DA) and serotonin (5-hydroxytryptamine, 5-HT) in prefrontal cortex by HPLC analysis.
Results: GHRHKO mice showed increased exploratory activity. In the open field test ($P < 0.005$), light–dark box ($P < 0.005$) and elevated plus maze ($P < 0.05$), GHRHKO mice demonstrated a decrease in anxiety-related behaviour. In addition, GHRHKO mice showed reduced immobility time with respect to control in forced swim test and tail suspension test ($P < 0.0001$). The gene expression of hypothalamic TRH ($P < 0.05$) was increased, while NE levels in prefrontal cortex were decreased compared to control ($P < 0.05$).
Conclusion: These results suggest that in male mice GHRH deficiency brings about an increased physical activity and decreased anxiety- and depression-related behaviour, possibly related to increased TRH and decreased NE levels in the brain.

1. Introduction

The role of GH in anxiety is still not well defined. The results of human studies indicate that in adults with childhood onset GH deficiency long-term treatment with GH improves mood and memory [1]. On the other hand, somatostatin (which inhibits the release of several hormones, including GH) reduces anxiety-like behaviour [2,3]. Furthermore, in rats somatostatin increases ambulation in the open field test [4]. Recent mouse studies have also demonstrated that 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 [5]. GHRH is a powerful and necessary stimulus for GH secretion. Ablation of GHRH results in somatotroph cells hypoplasia and severe GH deficiency [6]. Mice with targeted disruption (knock-out, KO) of the GHRH gene (GHRHKO) have been recognized as a model of isolated GH deficiency, and we have previously reported endocrine and behavioural alterations in this animal model [6,7]. The aim of present work was to further elucidate the role of GHRH and GH deficiency in emotional behaviour in these mice, with a series of validated behavioural tests, such as locomotor activity/open field test, light–dark exploration test, elevated plus-maze test, forced swim test and tail suspension test. We also correlated the behavioural results with neurotransmitters that have been implicated in behavioural functions in the brain: hypothalamic thyrotropin-releasing hormone (TRH), and pre-frontal cortex dopamine (DA), norepinephrine (NE) and serotonin (5-hydroxytryptamine, 5-HT).

2. Materials and methods

2.1. Animals

Homozygous mice carrying targeted ablation of the GHRH gene were previously described [6]. We used adult (3 month old at the beginning of

Abbreviations: DA, dopamine; GH, growth hormone; GHRH, growth hormone-releasing hormone; 5-HT, serotonin (5-hydroxytryptamine); IGF-I, insulin-like growth factor; KO, knock-out; NE, norepinephrine; TRH, thyrotropin-releasing hormone; WT, wild type.

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the experimental procedures) male (10–15 g) homozygous GHRHKO mice ($n = 12$) and adult (3 month old at the beginning of the experimental procedures) male wild type (WT) mice (20–25 g) ($n = 12$) as control group. GHRHKO offspring was generated by mating heterozygous males and females, as previously reported [6]. Only male mice were used to avoid any possible involvement of hormonal changes in adult female mice. The animals were housed in Plexiglas cages (2–3 animals per cage; 55 × 33 × 19) and maintained under standard laboratory conditions (21 ± 2 °C; 55 ± 5% humidity) on a 14/10 h light/dark cycle, with *ad libitum* access to water and food. The mice were fed with a standard rodent chow (Prolab RMH2500, PMI Nutrition International, Brentwood, MO). Housing conditions and experimentation procedures were strictly in accordance with the European Community ethical regulations on the care of animals for scientific research, and were approved by the Ethical Committee on Animal Health and Care of G. d'Annunzio University.

2.2. Behavioural test battery

GHRHKO mice were compared with WT mice using a battery of behavioural tests in the following order: locomotor activity/open field test, light–dark exploration test, elevated plus-maze test, forced swim test and tail suspension test. The tests were spaced by a 3–6-day inter-test interval, and were adapted from previously published reports [8,9] showing that this study design does not affect behavioural test performance. The animals were brought into the experimental room 30 min prior to the test in order for them to acclimate to the environment, and were kept in the testing chamber for 5 min prior to each test. Experiments were performed between 0900 am and 1200 pm and the test session was recorded by a video camera connected to a computer; a single video frame was acquired with a highly accurate, programmable, monochrome frame grabber board (Data Translation™, type DT3153). The intelligent software Smart version 2.5 (Panlab, sl Bioresearch and Technology, Barcelona, Spain) was used for data processing. The apparatuses were purchased from 2 Biological Instruments (Besozzo VA, Italy). At the end of each test, the animal was returned to its home cage, and the apparatus was cleaned with 75% ethanol and dried before the next animal was tested. After completion of all behavioural tests (about 45 days after the beginning of the randomization of the mice for the experimental procedures), the animals were sacrificed by decapitation, after Halothane overdose, as previously reported [7], 2 h after the last test.

2.2.1. Locomotor activity/open field test

Locomotor activity was recorded in the home cage over 10 min. The activity monitor consisted of a black and white video camera, mounted in the top-centre of a cage (35 × 20 × 13 cm), positioned in the enclosure. Measurements used to assess locomotor activity were horizontal activity, vertical activity, and total distance travelled.

To evaluate anxiety-like behaviour, each animal was placed in an open field box (40 × 40 × 31 cm) made of clear Plexiglas 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 the observation chamber were recorded.

2.2.2. Light–dark exploration test

The light–dark box test assesses bright-space related anxiety [10] and consists of two compartments (10 × 15 × 20 cm, each), dark and light ones, separated by a wall pierced with an open door. The dark compartment has opaque black walls, while the light compartment is transparent to light. Mice were placed in the black compartment, and time spent by the animal in the light compartment, latency of first exit from dark compartment, and number of transitions between compartments were recorded during a 10 min interval.

2.2.3. 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 [11]. The time spent on open arms, the latency to first exit and the number of transitions between the arms were recorded during a 10 min test period.

2.2.4. Forced swim test

The forced swim test was based on the original version of the Porsolt forced swim test for mice [12] 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 ± 1 °C to avoid severe hypothermia [13]. The total time that mice spent remaining immobile was measured. Immobility was determined when the mouse was only marking 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. On day 1, 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. On day 2 the mice were placed in water through a series of four trials of 6 min each and immobility was recorded during the last 4 min of each trial. Each trial was followed by an 8 min rest period when the animals were dried with towels and returned to their cage. Water in the cylinder was changed for each mouse.

2.2.5. Tail suspension test

This test is well characterized for assessing antidepressant-like activity [12,14]. Mice were individually suspended by the tail to a horizontal bar (30 cm height from floor) using adhesive tape for 10 min. Typically, mice demonstrate several escape-orientated behaviours interspersed with temporally increasing bouts of immobility. The number of seconds spent immobile was recorded during the last 4 min.

2.3. RNA extraction

Upon completing the behavioural tests, the animals were sacrificed. Hypothalami were rapidly dissected and stored in RNA*later* solution (Ambion, Austin, TX) at −20 °C until further processed. Total RNA was extracted using TRI Reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. In preliminary experiments performed in our laboratories on mice of the same age and weight, we have stereotaxically confirmed the location of the hypothalamus by the injection of dye (Evans blue 0.5% and Zelatin 5%) and histological examinations of frozen hypothalamic sections.

The RNA solution was quantified at 260 nm by spectrophotometer reading (BioPhotometer, Eppendorf, Hamburg, Germany) and its purity was assessed by the ratio at 260 and 280 nm readings. The quality of the extracted RNA samples was also determined by electrophoresis through agarose gels and staining with ethidium bromide, under UV light.

2.4. Reverse transcription and real-time reverse transcription polymerase chain reaction (real-time RT PCR)

1 μg of total RNA extracted from each sample in a 20 μl reaction volume was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's manual. Reactions were incubated in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) initially at 25 °C for 10 min, then at 37 °C for 120 min and finally at 85 °C for 5 s.

Gene expression was determined by quantitative real-time PCR using TaqMan probe-based chemistry (Applied Biosystems, Foster City, CA). Reactions were performed in MicroAmp Fast Optic 96-well Reaction Plates (Applied Biosystems, Foster City, CA) on an ABI PRISM