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Effects of GH deficiency and GH replacement on inter-male aggressiveness in mice

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ABSTRACT

Objective: Growth hormone (GH) has been suggested to influence aggressive behavior in several species, but no data are presently available in GH-deficient (GHD) animals. The aim of this study was to elucidate the effects of GHD on aggressive behavior in a mouse model of isolated GHD due to removal of the GHRH gene (GHRH knock out, GHRHKO), and to evaluate the effects of GH replacement.

Design: We studied two groups of adult male mice: Ten GH-sufficient animals heterozygous for GHRHKO allele (HTZ), and 30 GHRHKO animals. Behavior was measured by scoring several aggression parameters after isolation, when the animal was challenged against an intruder both in neutral and home cage. Animals were then re-studied after the GHRHKO mice were left untreated (control, Ctrl), or were treated for 2 weeks with daily subcutaneous recombinant GH or with vehicle (Veh). Blood samples were collected before and after GH or Veh treatment, and assayed for serum IGF-I and testosterone.

Results: The GHRHKO mice showed significantly reduced aggressiveness compared to HTZ animals. GH (but not Veh) administration normalized isolation-induced aggressive behavior in GHRHKO mice, despite lack of full serum IGF-I normalization. No difference was noted in serum testosterone levels among all groups at any of the time points.

Conclusions: These findings show that GHD reduces aggressive behavior in GHRHKO mice, that GH replacement normalizes aggressiveness, and that this behavior change is not related to an increase in serum testosterone.

1. Introduction

Growth hormone (GH) stimulates somatic growth, and has a key role in metabolism, reproduction, and lactation [1,2]. GH acts both directly and indirectly through circulating and locally produced insulin-like growth factor-I (IGF-I) [3–5]. GH binding sites have been demonstrated in the central nervous system suggesting that GH may also have a direct action on the brain. Accordingly, GH has been shown to influence social behavior, sleep, learning, and memory in vertebrates [6–10]. In humans, both GH deficiency and GH excess (as seen in patients with GH-secreting adenomas) significantly affect self-perceived quality of life [11].

Aggression is defined as any behavior by which one animal attacks or bites another animal. In particular, male mice often show aggressive behavior against novel males for territorial competition. Aggressive behavior is affected by a variety of physiological and environmental factors. Neural circuits regulating aggression have been identified in a variety of species [12], but very little is known about how aggressiveness is affected by the hormonal milieu. Pituitary hormones such as adrenocorticotropic hormone, thyroid-stimulating hormone, and gonadotropic hormones have been shown to influence aggressiveness in mice, likely through their effect on the respective target glands [13,14], but few and controversial data exist about the role of GH. In juvenile rainbow trout fish GH treatment increases appetite, swimming, and aggression [9,10]. Similar results are seen in GH transgenic salmon [15,16]. On the contrary, GH administered directly into the third ventricle increases swimming and locomotion, but not feeding, suggesting a different effect of GH on CNS depending on the route of administration [17,18]. In GH sufficient mice, exogenous GH administration increases isolation-induced aggression, without affecting non-aggressive motor activity [19]. Conversely, GH treatment decreases motor activity in rats [6,20]. GH also significantly influences aggressive behavior in young ruminant species [21]. No data is presently available on the aggressive behavior of GH-deficient (GHD) animals. We have recently developed a mouse model of isolated GHD by generalized ablation (knock-out, KO) of the GHRH gene that is necessary for the synthesis and secretion of GH (GHRHKO) [22]. The mice homozygous for the GHRHKO allele have proportionate dwarfism, with body size that is approximately 60% of non-GHD littermates. They are otherwise normal, with normal fertility and sexual behavior. We have noted that GHRHKO mice appear more docile and less aggressive than normal mice, with

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reduced combativeness and biting behavior when manipulated by investigators, and when interacting with other mice. Therefore, we decided to study the aggressive behavior of these mice, analyzing inter-male aggression by comparing GHRHKO mice with GH-sufficient mice with similar genetic background. We also investigated the effects of two weeks of GH replacement.

2. Material and methods

2.1. Animals and growth hormone administration

Aggressive behavior is divided according to the stimulus that provokes the behavior in: a) predatory aggression (in the presence of a natural prey); b) inter-male aggression (in the presence of a novel male in a neutral arena); c) fear-induced aggression (presence of any attackable object after isolation); d) territorial aggression (in the presence of an intruder in the proximity of the dam's young); and e) instrumental aggression (caused by any of the situation already described but strengthened by learning) [23]. For the characterization of aggressive behavior in male mice, different test paradigms that study b) and c) are commonly used. We used male GHRHKO mice born from homozygous GHRHKO parent to obviate the need for early genotyping. In order to maintain similar genetic background (mixed C57BL6 and 129SV) mice heterozygous (HTZ) for the GHRHKO allele, born from male GHRHKO animals and wild type females were used as normal size controls. All pups were weaned at the 4th week of age. Four groups of adult animals (10 mice each) (3 groups of GHRHKO and 1 HTZ) were studied: one group of GHRHKO was left untreated (Ctrl), one received GH therapy (GH), and one received vehicle injections (Veh). Heterozygous GH-sufficient mice (HTZ) were not treated. Following guidance from a previous study aimed to normalize growth [24], the GH group received 70 μg/daily of porcine GH subcutaneously from the 14th to 16th week of age while the Veh group received similar volume (53 μl) of 0.9% NaCl. Porcine GH has been shown to be effective in mice [25]. All mice experienced a controlled environment with 14 h light/10 h dark cycles at 21 °C and 23% humidity, food standard mouse/rat (Prolab RMH2500, PMI Nutrition International, Brentwood, MO) and water *ad libitum.* All procedures were approved by the Johns Hopkins Institutional Animal Care Committee.

2.2. Animal behavior tests

After weaning, pups were grouped until the 10th week of age, when spontaneous aggression was tested in a neutral cage (Test A). Mice were then isolated in individual cages until sacrifice, as previously reported [26]. After the initial 2 weeks of isolation (12th week of age), aggression was tested in a neutral cage, where the tested mouse and a male opponent were placed simultaneously in the opposite corners and fixed there for 5 s before the start of the testing (Test B) [27]. After 2 additional weeks of isolation (14th week), the experiment was repeated in the cage where the mouse was in isolation (Test C) (territorial aggression test) [28]. Before the standard opponent was taken to the test location, the littermates of the test animal were removed and the resident mouse was fixed in one corner.

Tests B and C were then repeated at the 16th week of age, after 2 groups of animals received treatment (GH or Veh). Tests were performed 6–8 h after the last GH or vehicle injection. In order to maintain weight matching, peaceful appearing GHRHKO opponent were used for the GHRHKO mice test, and HTZ opponents for the HTZ mice. In all the experiments, the tested animal was videotaped for 10 min after introduction of the opponent mouse, and its behavior was then analyzed. Five parameters were considered: duration of ano-genital investigation (AGD), duration of rough grooming (RGD), frequency of biting attacks (BAF), tail-rattling frequency (TRF), and threat posture frequency (THF). The test is divided into 4 intervals, where the first 2 intervals come before the onset of the attack and last maximum 150 s each. If the latency to attack is less than 5 min the pre-attack interval is divided into 2 parts. If the latency to attack is greater than 5 min, the test is scored zero. The duration of the fighting intervals is 150 s each. The test duration went from 5 (minimum) to 10 (maximum) min. The total aggressive score was calculated according to previously published literature [27], after normalizing each parameter to per minute scores (the number after the three letter codes identifying the behavior refers to the corresponding interval). $(\text{AGGRESSION SCORE} = 0.03(\text{AGD2}) - 0.04(\text{RGD2}) - 0.07(\text{RGD3}) + 0.31\ (\text{TRF3}) + 0.41(\text{BAF3}) - 0.07(\text{RGD4}) + 0.51(\text{TRF4}) + 0.39(\text{THF4}) + 0.38(\text{BAF4})$ [26]. All tests were evaluated by a single investigator (AS) at the end of the study, who was blinded to which treatment group the GHRHKO animals belonged to (blinding was not possible for HTZ animals, which appear larger than GHRHKO).

At 14th and 16th week of age approximately 100 μl of blood was collected with heparinized capillaries by retro-orbital bleed for testosterone and IGF-I measurements. Sera were stored at −80 C until the day of assay. After completion of the experiments, mice were sacrificed by Halothane overdose, measured, and weighed. Heart, liver, spleen, right kidney, right lung, right testis and seminal vesicles were harvested and weighed using an Ohaus Adventurer Pro Analytical Balance (AV264 Ohaus, Pine Brook, NJ).

2.3. Serum hormone measurements

Serum IGF-I and testosterone were measured by commercially available rodent-specific ELISA kits (22-IG1MS-E01 and 11-TESHU-E01, ALPCO Diagnostics, Salem, NH) according to the manufacturer's instruction manual. Samples were analyzed in duplicate and the hormone levels were measured according to the calibration curves established from standards.

The sensitivity of the ELISA assays was 0.022 ng/ml for testosterone and 0.029 ng/ml for IGF-I. Inter and intra-assay variability were <5% for both assays.

2.4. Statistical analysis

Data analysis comparing hormonal levels in different groups at the same time points and weights and lengths at the time of sacrifice was performed by one-way analysis of variance (ANOVA), using the SPSS statistical package (SPSS, Chicago, IL), with post hoc analysis according to the Bonferroni method. Percents of mice showing aggressive behavior were compared using Fisher's exact test. Changes in serum IGF-1 within groups between 14th and 16th week were analyzed by paired t-test. Data were considered statistically significant at $p<0.05$.

3. Results

3.1. Animal behavior tests

At 10th week (before isolation) only 1 of 10 HTZ mice and none of the 30 GHRHKO animals showed aggressive behavior against opponent mouse.

After 2 weeks of isolation (age 12 weeks) on test B (neutral cage) 4 of 10 HTZ mice demonstrated aggressive behavior with mean scores of 0.27 ± 0.32, while only 2 of the 30 GHRHKO animals showed aggressive behavior (1 in the GH and 1 in the Ctrl group) ($p<0.05$ by Fisher's exact test). After 2 weeks, on test C (home cage), 6 HTZ (and none of the 30 GHRHKO animals) showed aggressive behavior with an average score of 0.47 ± 0.42.

At 16 weeks of age (after a total of 6 weeks of isolation and GH or vehicle treatment), on test B (neutral cage), 3 of the 10 HTZ mice showed aggressive behavior with an average score of 0.15 ± 0.24, while this occurred only in 2 GHRHKO animals, one in the GH-treated group, and one in the Ctrl group.