0013-7227/00/$03.00/0
Endocrinology
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Vol. 141, No. 2
Printed in U.S.A.

Neuroendocrine Function and Response to Stress in Mice with Complete Disruption of Glucagon-Like Peptide-1 Receptor Signaling*

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ABSTRACT

Glucagon-like peptide-1 (GLP-1), a potent regulator of glucose homeostasis, is also produced in the central nervous system, where GLP-1 has been implicated in the neuroendocrine control of hypothalamic-pituitary function, food intake, and the response to stress. The finding that intracerebroventricular GLP-1 stimulates LH, TSH, corticosterone, and vasopressin secretion in rats prompted us to assess the neuroendocrine consequences of disrupting GLP-1 signaling in mice *in vivo*. Male GLP-1 receptor knockout (GLP-1R−/−) mice exhibit reduced gonadal weights, and females exhibit a slight delay in the onset of puberty; however, male and female GLP-1R−/− animals reproduce successfully and respond appropriately to fluid restriction. Although adrenal weights are reduced in GLP-1R−/− mice, hypothalamic CRH gene expression and circulating levels of corticosterone, thyroid hormone, testosterone, estradiol, and progesterone are normal in the absence of GLP-1R−/− signaling. Intriguingly, GLP-1R−/− mice exhibit paradoxically increased corticosterone responses to stress as well as abnormal responses to acoustic startle that are corrected by glucocorticoid treatment. These findings suggest that although GLP-1R signaling is not essential for development and basal function of the murine hypothalamic-pituitary-adrenal axis, abrogation of GLP-1 signaling is associated with impairment of the behavioral and neuroendocrine responses to stress. (*Endocrinology* **141:** 752–762, 2000)

IN MAMMALS, a single proglucagon gene encodes several structurally related glucagon-like peptides (GLPs) that are liberated from a common prohormone precursor by tissue-specific expression of prohormone convertase enzymes in pancreas, intestine, and brain. Proglucagon processing in the pancreas generates 29-amino acid glucagon in the pancreatic A cells, which functions as a key counterregulatory hormone important for the control of hepatic glucose production *in vivo*. In contrast, proglucagon cleavage in the intestine yields several proglucagon-derived peptides (PGDPs), including glicentin, oxyntomodulin, GLP-1, and GLP-2 (1). Although glicentin and oxyntomodulin have been shown to regulate acid secretion and intestinal glucose uptake (2), GLP-1 appears to be the gut-derived PGDP with more potent actions on gastric emptying and glycemic control (1).

Considerable evidence supports multiple complementary biological actions for GLP-1 in the control of glucose homeostasis. Nutrient intake promotes GLP-1 release from the intestine, which serves to stimulate glucose-dependent insulin secretion from the pancreatic β-cell (3–5). GLP-1 also inhibits glucagon secretion and decreases gastric emptying, which further reduce glycemic excursion *in vivo*. Taken together, these peripheral actions of GLP-1 function promote regulated nutrient assimilation and glucose control in both normal subjects and patients with type 2 diabetes (1).

The role of the PGDPs in the central nervous system (CNS) is less well understood. Original observations demonstrating that GLP-1 stimulates adenylate cyclase activity in hypothalamic and pituitary membrane preparations (6) were followed by the detection of CNS proglucagon messenger RNA (mRNA) transcripts, predominantly in the brain stem (7, 8). After the cloning of distinct PGDP receptors for glucagon, GLP-1, and GLP-2, a combination of molecular techniques has localized the expression of all three PGDP receptors to different regions of the rodent and human CNS (9–11). These findings have stimulated additional interest in the putative roles of the PGDPs in neuroendocrine systems *in vivo*.

Considerable experimental data support one or more actions for GLP-1 in the CNS. Intracerebroventricular (icv) administration of GLP-1 inhibits food and water intake in rats and mice (12, 13). Conversely, administration of the GLP-1 receptor antagonist exendin-(9–39) promotes increased food intake in acute, short term studies and increased weight gain in more long term, chronic experiments in rats (12, 14). Whether GLP-1 acts directly to inhibit food intake or induces conditioned taste aversion leading to decreased satiety (15) remains unclear. However, GLP-1 does not appear

Received September 14, 1999.

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* This work was supported in part by operating grants from the Juvenile Diabetes Foundation International and the Canadian Diabetes Association.

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to be essential for appetite regulation and weight control, as disruption of GLP-1 signaling in mice does not result in increased food intake or long term changes in body weight (13, 16).

More recent studies have suggested that GLP-1 may modulate the activity of the hypothalamic-pituitary axis. GLP-1 increased GnRH secretion from hypothalamic GT-1 cells, and icv injection of GLP-1 increased the levels of plasma LH in rats (17). Furthermore, icv injection of GLP-1 activated c-*fos* in CRH-positive neurons of the hypothalamic paraventricular nucleus in association with increased levels of plasma corticosterone in rats (18). These findings taken together with studies demonstrating GLP-1 regulation of pituitary TSH release (19) raise the possibility that GLP-1 may modulate hypothalamic-pituitary function at several levels *in vivo*.

Despite accumulating data suggesting a possible therapeutic role for GLP-1 in patients with type 2 diabetes, the physiological role of GLP-1 in the hypothalamic-pituitary axis remains uncertain. As the majority of previous studies examining this issue have used direct icv or iv administration of pharmacological amounts of GLP-1, the possibility cannot be excluded that such injections may exert nonphysiological effects, possibly via activation of related receptors or nonspecific anorectic or stress responses (20, 21). We have recently examined the phenotypic consequences of disrupting GLP-1 action *in vivo*, through generation and analysis of GLP-1 receptor knockout (GLP-1R−/−) mice. These mice are viable, but exhibit abnormal glucose homeostasis due to defective glucose-induced insulin secretion. Importantly, GLP-1R−/− mice do not respond to GLP-1 administration, and no GLP-1-binding sites have been detected in the pancreatic islet cells or CNS of GLP-1R−/− mice (13, 22). In the present study we have examined whether the specific loss of GLP-1 signaling is associated with perturbations in regulation of neuroendocrine function *in vivo*.

Materials and Methods

Animals

GLP-1R−/− mice were generated by targeted disruption of the gene encoding the GLP-1R in embryonic stem cells (13, 22). Male and female CD1+/+ control mice and age- and sex-matched GLP-1R−/− mice were housed and cared for under standard environment conditions (five per cage). In one behavioral experiment, described below, male heterozygous GLP-1R+/− animals were also analyzed. All experiments were approved by the animal care committee of the Toronto Hospital. For breeding, two females were housed with one male for 4 weeks, then subsequently removed and housed individually. At parturition, the number of pups was counted, and pups were allowed to remain in their respective cages to be nursed by their mothers until weaning (3 weeks postpartum). Purina rat chow (Ralston Purina Co., St. Louis, MO) and water were freely accessible, and a 12-h light, 12-h dark cycle was maintained, with lights on at 0700 h. For analysis of reproductive function as well as for the majority of studies on regulation of corticosterone levels, experiments were carried out on littermate+/+ and GLP-1R−/− mice born and raised in the Toronto General Hospital (TGH) animal facilities. For other studies, age- and sex-matched CD1 control mice were either born at TGH or purchased from Charles River Laboratories, Inc. Canada (Toronto, Canada) and acclimatized to the TGH animal facility for at least 2 weeks before experimental analysis.

Body weights were measured from weaning until adulthood (~6 weeks of age). Females were inspected daily for vaginal opening as a biological index of the onset of the first estrous cycle. Vaginal smears were taken daily at 0900 h for a period of 24 days (six complete estrous cycles) to monitor cyclicity. Smears were obtained by flushing 50 μl deionized double distilled water gently into the vagina and immediately retrieving the water with a plastic pipette tip. Droplets of the recovered fluid were smeared onto precleaned glass slides and dried for microscopic examination. Females showing regular 4-day cyclic vaginal smear patterns were killed between 1000–1200 h on the appropriate days of the cycle by asphyxiation in a chamber filled with CO_2 gas followed immediately by exsanguination via cardiac puncture using a 23-gauge needle and decapitation. Blood was allowed to clot in a cold room at 4 C, and serum was separated by centrifugation for 1 min in a microfuge and stored frozen at −20 C. An additional group of female mice was allowed to remain in the TGH animal care facility until they were 90–100 days of age before vaginal smearing was initiated, to determine whether cyclicity was maintained in GLP-1R−/− animals. Approximately 1 month later (at 120–125 days of age) the animals were killed, serum was collected, and the ovaries were processed for histological examination.

At death in the majority of cases, male and female gonads and steroid-dependent accessory sex glands (epididymis, prostate gland, seminal vesicle, and uteri) were dissected free of surrounding fatty tissue, frozen over a sheet of aluminum foil covering dry ice, and weighed on a Sartorius Supermicro Balance (Sartorius, Goettingen, Germany). Where tissues were to be examined histologically, the organs were placed immediately after removal in buffered formalin solution and fixed overnight at room temperature before embedding in paraffin. Sections (4 μm) were prepared, stained with hematoxylin and eosin, and examined under a light microscope at ×100 magnification. Counts of numbers of antral follicles and corpora lutea were performed in full thickness sections prepared from ovaries from animals killed at the proestrous stage of the estrous cycle.

Because of the likelihood that circulating corticosterone levels would be influenced by the degree of stress to which the animals were exposed before death, groups of male mice were killed for serum corticosterone determination under four different conditions: 1) nonstressed: animals were withdrawn one by one from their home cages and killed immediately in the animal room; 2) environmental stress A: animals were removed from their home cages, transported in a holding cage approximately 50 ft down a corridor to the necropsy laboratory, and killed there, approximately 2–4 min elapsing between removal from the animal room and death; 3) environmental stress B: animals were removed from the animal care facilities in their home cages, transported up five floors in an elevator to laboratories on the fourth floor of the Max Bell Research Center of the Toronto Hospital Research Institute and killed there, approximately 1–2 h after arrival; and 4) anesthetic stress: mice were anesthetized with metofane, and 10 min later while they were still under anesthesia, blood was collected.

To examine the regulation of plasma osmolarity, age- and sex-matched GLP-1R−/− and wild-type+/+ mice were injected sc for 7 days with either 200 μl vehicle (saline) or hydrocortisone (10 mg) and subsequently either permitted access to drinking water *ad libitum* or denied access to water for the last 13 h before death. At death, blood was withdrawn via cardiac puncture into heparanized tubes, and plasma was separated by centrifugation. Plasma osmolarity was assessed using a vapor pressure osmometer (Wescor, Inc., Logan, UT).

RIAs

Serum T_4 was assayed using a kit purchased from ICN Biomedicals, Inc. (catalogue no. 06B254011; Montreal, Canada). Testosterone, progesterone, estradiol, and corticosterone levels were measured using kits purchased from Diagnostic Products (Los Angeles, CA; catalogue no. TKTT1, KE2D1, TKPG1, and TKRC1, respectively). For the estradiol assay, 100- to 200-μl aliquots of serum were extracted into 2 ml fresh anhydrous diethyl ether, evaporated to dryness, and reconstituted in 0.1% human serum albumin to eliminate interfering substances present in rodent serum. The remaining hormones were assayed directly in serum, according to the kit instructions.

Immunocytochemistry

Immunocytochemistry was performed on the anterior pituitaries of GLP-1R−/− and GLP-1R+/+ mice to determine the distribution and relative abundance of each of the major adenohypophyseal cell types as follows. Paraffin-embedded sections (4–5 μm thick) were stained with hematoxylin and eosin as well with the Gordon-Sweet silver method to

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demonstrate the reticulin fiber network. Immunocytochemical stains to localize adenohypophysial hormones were performed using the streptavidin-biotin-peroxidase complex technique. Primary antisera directed against rat pituitary hormones were used at the specified dilutions with overnight incubations: GH, 1:2500; PRL, 1:2500; TSHβ, 1:3000; FSHβ, 1:600; LHβ, 1:2500 (National Hormone and Pituitary Program, Rockville, MD); and ACTH prediluted preparation, further diluted 1:20 (DAKO Corp., Carpinteria, CA). The reaction products were visualized with the Ultrastreptavidin kit (Signet Laboratories, Inc., Dedham, MA) and 3,3′-diaminobenzidine.

In situ hybridization histochemistry

In situ hybridization histochemistry for hypothalamic CRH mRNA was performed on adult male GLP-1R−/− and GLP-1R+/+ mice, 7–9 weeks of age. Four experimental groups of animals were employed: fed (*ad libitum* access to chow and water) wild-type mice, fed GLP-1R−/− mice, 24-h fasted wild-type mice (*ad libitum* access to water only), and 24-h fasted GLP-1R−/− mice. Antisense riboprobes were created using an *in vitro* transcription kit (Promega Corp., Madison, WI) using complementary DNA for CRH (donated by Dr. Joel Elmquist). Sense and antisense complementary RNA probes were transcribed from 1 μg linearized DNA template, and *in situ* hybridization was carried out using [^{35}S]UTP-labeled complementary RNA probes as previously described (23).

Briefly, mice were deeply anesthetized with sodium pentobarbital (90 mg/kg BW, ip) at 1000 h. The animals were then perfused transcardially with 0.9% saline, followed by 10% neutral buffered formalin (Sigma, St. Louis, MO). The mice were fixed for 1 h at room temperature before the brains were removed and postfixed for additional 4 h in 10% neutral buffered formalin. Frozen sections (25 μm) in the coronal plane were obtained using a Reichert cryostat at −18 C. Consecutive sections through the paraventricular nucleus were collected from each brain and stored in cryoprotectant (5% neutral buffered formalin, 30% ethylene glycol, and 20% glycerol). Sections were mounted onto slides (SuperFrost Plus Slides, Fisher Scientific, Fairlawn, NJ) and stored at −20 C in an air-tight container until the *in situ* hybridization procedure (<1 week). Slides were postfixed in 37% (wt/vol) formaldehyde for 5 min, rinsed twice in 1 × PBS, incubated in 0.5 μg/ml proteinase K buffer [0.5 M EDTA, 1.0 M Tris (pH 8.0), and diethylpyrocarbonate-water] for 30 min at 37 C, rinsed with water and triethoalemine-HCl, acetylated with acetic anhydride (0.25%) in triethoalemine-HCl, dehydrated in an ethanol series (70%, 80%, 95%, and 100% ethanol; chloroform; and 100% and 95% ethanol), air-dried, and stored at −20 C until hybridization.

Sections were hybridized with [^{35}S]CRH riboprobe in a hybridization buffer [50% formamide, 0.1% SDS, 0.01% thiosulfate, 0.1 M dithiothreitol, 0.6 M NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 5% dextran sulfate, 0.01% sheared salmon sperm DNA, 0.05% total yeast transfer RNA, 0.01% yeast transfer RNA, and 1× Denhardt's solution] in a hybridization chamber consisting of tissue culture trays (VWR, Bridgeport, NJ), lined with chromatography paper saturated with a 50% formamide-4 × SSC (standard saline citrate) solution. The chamber was placed in a 58 C oven for 18.5 h. The slides were then washed four times in 2 × SSC and treated with ribonuclease A (20 μg/ml) for 30 min at 37 C. The sections were rinsed in buffer containing 0.5 M NaCl, 40 mM Tris-HCl (pH 8.0), and 1 mM EDTA (pH 8.0), then washed in 300 mM NaCl, 30 mM citrate (2 × saline-sodium citrate solution or 2 × SSC) and 1 M dithiothreitol (DTT) for 1 h at 50 C; 0.2 × SSC and 1 M DTT for 1 h at 55 C; and 0.2 × SSC and 1 M DTT for 1 h at 60 C. The slides were dehydrated in ethanol containing 0.3 M ammonium acetate and 1 mM DTT, air-dried for 1 h, and exposed to Biomax MR film (Eastman Kodak Co., Rochester, NY) for 48 h together with a ^{14}C-labeled standard (Amersham Pharmacia Biotech, Markham, Canada) to verify the linearity of the film response and the consistency of signal detection across films.

Slides were dipped in a 1:1 mixture of NTB2 emulsion-water (Kodak) in light-tight and high humidity conditions, dried overnight, and exposed at 4 C for 2 weeks. The slides were developed, counterstained with thionin for 2–3 min, dehydrated in a 50%, 70%, and 95% ethanol series with acetic acid, two changes of 95% ethanol, two changes of 100% ethanol, and xylenes before coverslipping with Permount medium (Fisher Scientific). The emulsion-coated slides were analyzed using a Leitz light microscope (Rockleigh, NJ). Photomicrographs were generated by capturing images with a digital camera mounted directly on the microscope, and Apple Macintosh Image editing software (Apple Corp., Cupertino, CA) was used to transfer the images to a Power Point program for display.

Elevated plus maze test

The elevated plus maze consisted of two opposite open arms (50 × 10 cm) and two arms with walls (50 × 10 × 40 cm) attached to a central platform (10 × 10 cm) to form a cross and elevated 65 cm above the ground. The maze floor was constructed from white Plexiglas, and the walls were made of black Plexiglas. Black lines on the maze floor defined the center square and divided both the open and closed arms into two equal segments, designated close and far based on proximity to the center square. Tests were carried out during the light phase of the light-dark cycle. Testing commenced by placing each mouse on the central platform facing an open arm. The GLP-1R−/− and wild-type CD1 mice were tested in alternating order. After each 5-min test, the maze was cleaned. The behavior of the animals in the maze was videotaped under white light illumination. The number and duration of closed arm and open arm entries with both forepaws were recorded and analyzed as previously described (24).

Acoustic startle responses

Amplitudes of startle reactions were determined using an acoustic startle apparatus obtained from MED Associates (St. Albans, VT). The mouse startle chamber was housed within a sound-attenuated acoustic startle cubicle (54.6 × 50.8 × 30.5 cm, interior dimensions) with a ventilation fan. The chamber was constructed of stainless steel rods suspended between Plexiglas plates fixed to a Plexiglas base. Interior dimensions of the chamber were 5.1 × 3.8 × 3.8 cm, providing the animal some movement. The magnitude of the startle response was measured by the startle platform for a period of 500 msec from presentation of the acoustic stimuli and subsequently transduced and collected onto a PC using Startle Reflex Software for Windows (MED Associates). Acoustic white noise stimuli were presented through a programmable audio stimulator (MED Associates, St. Alban's, VT). Background white noise levels were maintained at 70 dB throughout testing. Sixteen-week-old male GLP-1R−/− (n = l0), age-matched wild-type CD1 (n = 11), and heterozygous GLP-1R+/− (n = 11) mice were tested in alternating order within the 4 acoustic startle chambers. Mice were placed in the startle chamber for a 5-min acclimation period with a background noise level of 70 dB. After the acclimation period, mice were exposed to acoustic startle intensities of 83, 85, 90, 100, and 120 dB for a duration of 30 msec in random order. Testing consisted of 40 trials of the 5 randomly presented stimulus intensities. Each mouse was therefore exposed to a total of 200 acoustic stimuli, or 40 at each of the 5 intensities. The startle amplitude at each stimulus intensity was defined as the average of 40 readings. The time interval between stimuli was varied, lasting from 12–18 sec. Five weeks after the initial startle testing, some of the same experimental groups of GLP-1R−/− (n = 8), GLP-1R+/− (n = 8), and GLP-1R+/+ (n = 8) mice received 0.5 mg/ml hydrocortisone sodium succinate (Abbott Laboratories, Saint-Laurent, Canada) in *ad libitum* drinking water for a period of 6 days, after which the acoustic startle test was repeated. After this final test, the animals were killed in the biochemistry laboratory, and serum was collected for corticosterone assay under conditions identical to those described for the environmental stress B group described above.

Statistical analysis

All data are presented as the mean ± SEM. For most experiments, statistical analysis was performed using PC-compatible microcomputer programs [SPSS for Windows (SPSS, Inc., Chicago, IL) and SigmaStat (Jandel Scientific, San Rafael, CA)]. The data from the maze tests were analyzed on an Apple MacIntosh computer using INSTAT 1.12 (GraphPad Software, Inc., San Diego, CA). Two group comparisons were made using unpaired Student's *t* tests. Multiple group comparisons were made using ANOVA, followed by the Newman-Keuls test for individual group comparisons. Log transformation of the data were used to eliminate inhomogeneity of variance where necessary.

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Results

Growth rates, sex differences, and organ weights

Adult male GLP-1R−/− mice were normal in size and body weight and appeared phenotypically indistinguishable from GLP-1R+/+ controls (Fig. 1, A and B). In contrast, adult female GLP-1R−/− mice were slightly but significantly lighter than GLP-1R+/+ female controls (Fig. 1C), consistent with the results of previous studies (13, 16, 25). The animals were fertile, with no obvious reproductive deficits.

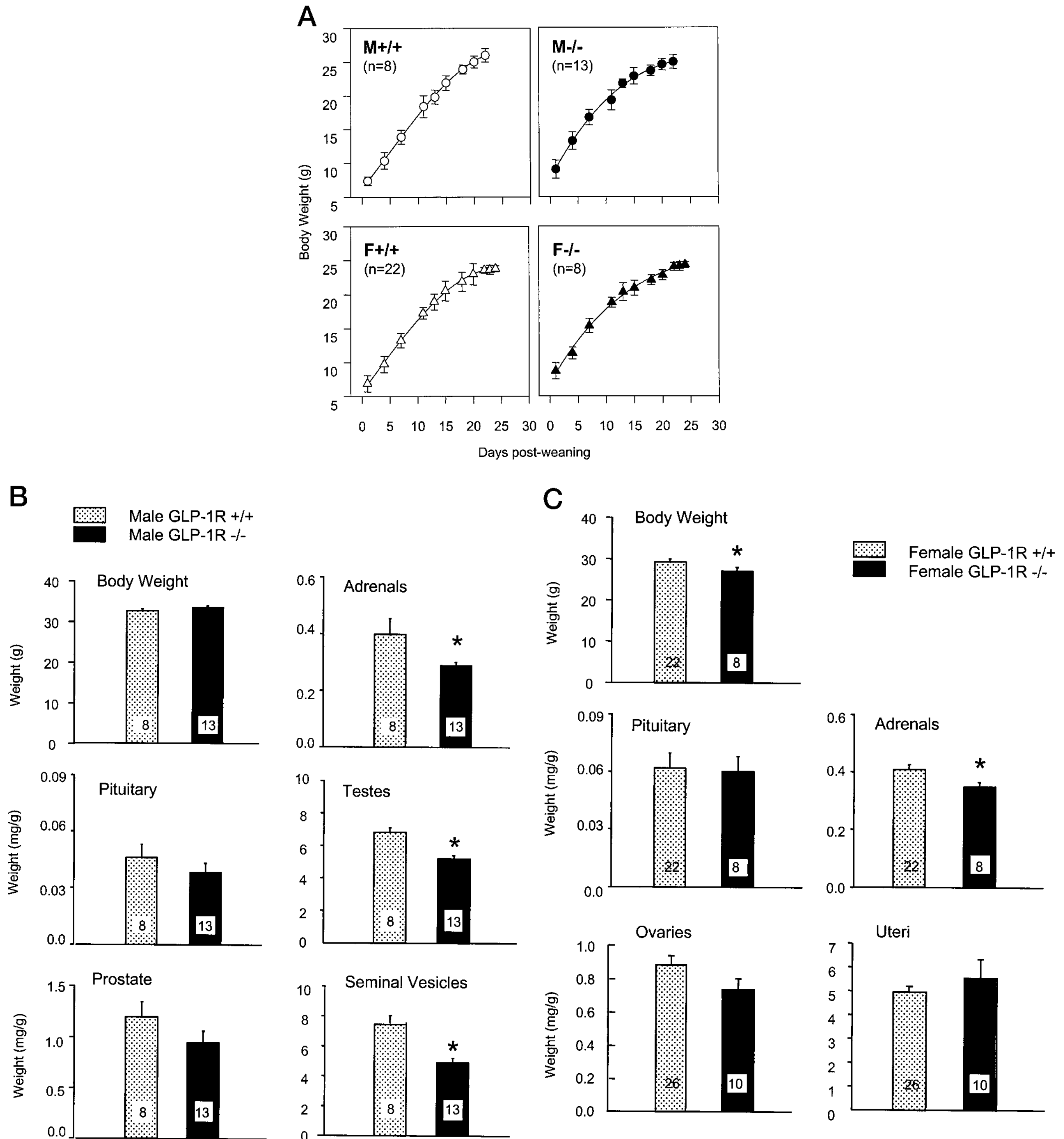

FIG. 1. A, Body weight gain in male and female GLP-1R+/+ and GLP-1R−/− mice born in the TGH animal facility and raised under identical housing conditions. B and C, Body and tissue weights in 6-week-old male and female GLP-1R+/+ and GLP-1R−/− mice. The number (n) of experimental observations for each measurement is shown in each panel. *, $P < 0.05$, +/+ *vs.* −/− mice.

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Growth rates in littermate male and female mice, assessed from weaning to adulthood, showed the expected postpubertal sex differences, but were not significantly different between GLP-IR−/− animals and wild-type controls reared under identical conditions (Fig. 1A).

At autopsy, male GLP-1R−/− mice exhibited small, but significant, decreases in adrenal, testis, and seminal vesicle weights compared with age-matched control animals (Fig. 1B; $P < 0.05$, +/+ *vs.* −/− mice). Adrenal weights were also significantly reduced in female GLP-1R−/− mice. However, no statistically significant differences were observed in the weights of the ovaries or uteri of GLP-1R−/− *vs.* GLP-1R+/+ mice (Fig. 1C). Similarly, pituitary weights were comparable in male and female GLP-1R+/+ and GLP-1R−/− animals (Fig. 1, B and C). Histologically, we did not observe any qualitative changes in the hypothalamus (not shown) or pituitaries of GLP-1R−/− mice (Fig 2).

Immunocytochemical staining for each of the principal anterior pituitary hormones revealed a normal number and distribution of adenohypophyseal cell types, including corticotrophs and thyrotrophs (Fig. 2A) and gonadotrophs (not shown), in GLP-1R−/− mice. Despite the significant decrease in adrenal weights, adrenal histology was normal in GLP-1R−/− mice, with appropriate numbers of cell types and normal zonal distribution observed in adrenal glands from both male and female GLP-1R−/− mice (Fig. 2B). Similarly, despite a significant decrease in testicular weight, the histological architecture and cellular organization appeared comparable in GLP-1R+/+ and GLP-1R−/− mice even at 120–125 days of age (Fig. 2B). Although ovarian histology appeared grossly normal in GLP-1R−/− mice, a small, but significant, decrease in the mean number of developing follicles was observed in GLP-1R−/− ovaries (Fig. 3). The mean number of corpora lutea was also slightly lower in GLP-1R−/− mice, although this difference did not attain statistical significance.

To assess the functional integrity of the hypothalamic-pituitary gonadal axis in females, we initially determined the timing of puberty in littermate GLP-1R+/+ and GLP-1R−/− animals. Although the +/+ mice reached puberty (defined by vaginal opening and the onset of the first reproductive cycle) on postpartum day 38, a 2-day delay was consistently observed in GLP-1R−/− females (Fig. 3). Cyclicity in female GLP-1R−/− mice was normal once vaginal opening had occurred and was indistinguishable from that in female +/+ control mice. A total of 20 wild-type +/+ and 10 GLP-1R−/− females were studied. All exhibited regular 4-day estrous cycles throughout the 24 days of the initial smearing period. Of the animals that were allowed to reach 120–125 days of age, the great majority of both genotypes continued to exhibit regular 4-day estrous cycles until the day of death (GLP-1R−/− mice, 10 cycling and 1 acyclic; GLP-1R+/+ animals, 12 cycling and 1 acyclic). Both animals that stopped cycling exhibited persistent estrous vaginal smears, consistent with previous studies on hypothalamic failure in aging mice (26).

Hormone levels

In rats, GLP-1 may modulate LH, TSH, and CRH secretion (17–19). Therefore, we assessed the levels of thyroid hormone as well as the principal gonadal and adrenal steroids in GLP-1R+/+ and GLP-1R−/− mice. No differences in the levels of testosterone or thyroid hormone (T_4) were detected in age-matched GLP-1R+/+ and GLP-1R−/− males (Fig 4). Serum estradiol and progesterone measurements in cycling females were also comparable in GLP-1R+/+ and GLP-1R−/− animals. Although there was a trend toward lower progesterone levels in the GLP-1R−/− mice, this difference was not statistically significant on any individual day of the estrous cycle (Fig. 4). No significant differences in basal morning circulating corticosterone levels were observed between GLP-1R−/− and GLP-1R+/+ control mice of either sex despite the small, but significant, decrease in adrenal mass in GLP-1R−/− mice (Fig. 5, A and B). In contrast, corticosterone levels were significantly greater in male GLP-1R−/− mice killed under different stress paradigms (Fig. 5B).

CRH in situ hybridization

The finding that GLP-1R−/− mice exhibited normal basal corticosterone but increased corticosterone levels in response to novel environments or anesthetic stress suggested that the loss of GLP-1 signaling might have perturbed the development and/or function of the hypothalamic CRH system. To address this possibility, *in situ* hybridization was performed to examine the distribution of CRH mRNA-expressing neurons in the hypothalamic paraventricular nucleus. No significant differences in the number and distribution of CRH^+ neurons or in the relative level of hypothalamic CRH gene expression were observed in age- and sex-matched GLP-1R+/+ *vs.* GLP-1R−/− animals (Fig. 6). The highest density of labeled cells was observed in the lateral and medial magnocellular division, but many labeled cells were also seen in the medial parvocellular division. There were no significant differences in the intensity of the CRH signal in either fed (n = 4) or fasted (n = 4) GLP-1R+/+ *vs.* GLP-1R−/− mice (Fig. 6, A–D).

Behavioral studies

A number of previous studies have suggested that GLP-1 may regulate hypothalamic CRH and both water intake and vasopressin release (18, 27). Both of these hormones contribute to the regulation of stress-induced increases in adrenal cortical hormone secretion. Changes in intracerebral CRH and vasopressin concentrations also affect behavioral responses to changes in the internal and external environment, and vasopressin is critically important for the regulation of drinking behavior and, hence, plasma osmolarity. As acoustic startle responses and behavior in elevated plus maze tests are both altered after intracerebral administration of CRH or vasopressin, we used these paradigms to examine whether loss of GLP-1 signaling might lead to subtle impairment of CRH or vasopressin-dependent behaviors GLP-1R−/− mice.

To exclude the confounding variable of dehydration impacting on results of behavioral studies, plasma osmolarity was assessed. Osmolarity was normal in GLP-1R−/− mice and was slightly reduced after several days of glucocorticoid administration (Fig. 7A). Nevertheless, plasma osmolarity rose appropriately, but not excessively, after 13 h of water deprivation in both saline- and glucocorticoid-treated GLP-

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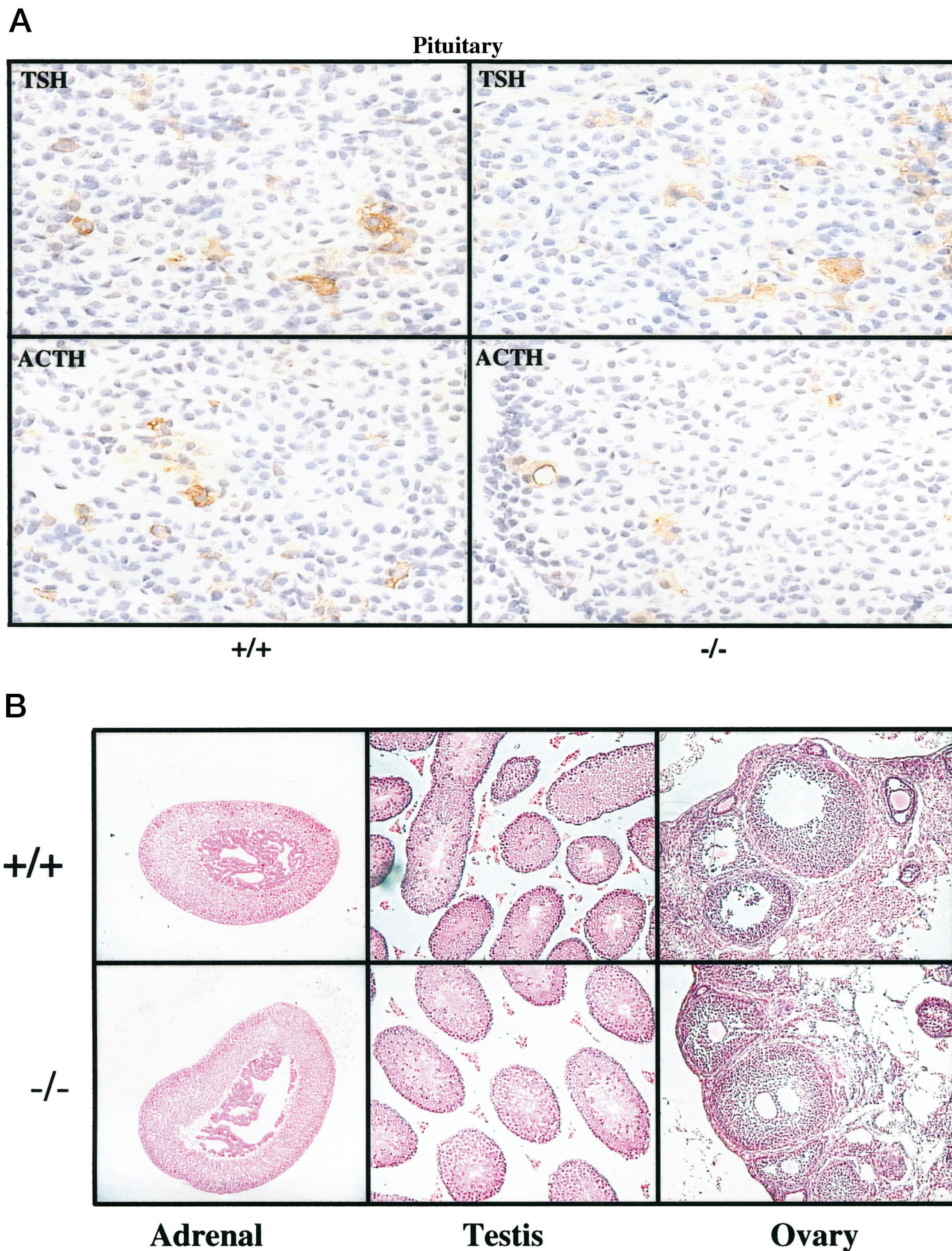

FIG. 2. A, GLP-1R−/− pituitary glands contain normal numbers and distribution of thyrotrophs (TSH) and corticotrophs (ACTH). +/+ and −/− denote age- and sex-matched wild-type and GLP-1R−/− mice, respectively. Magnification, ×20. B, Histological analysis of adrenal (×4), testis (×10), and ovary (×10) from GLP-1R+/+ and GLP-1R−/− mice.

1R−/− mice. These findings demonstrate that GLP-1R−/− mice do not exhibit basal abnormalities in osmoregulation and are able to sense and respond to fluid restriction, indicative of a functionally intact vasopressin response *in vivo* (Fig. 7A).

In the elevated plus maze, the percentage of open arm entries *vs.* closed arm entries was not significantly different between GLP-1R−/− and GLP-1R+/+ control mice (P = 0.13; Fig. 7B). However, GLP-1R−/− mice spent a significantly greater amount of time in the center square of the maze compared with wild-type controls ($P < 0.01$; Fig. 7C). Significant differences between the genotypes were also observed in terms of acoustic startle response. At low stimulus intensities (<100 dB), the GLP-1R−/− animals appeared to

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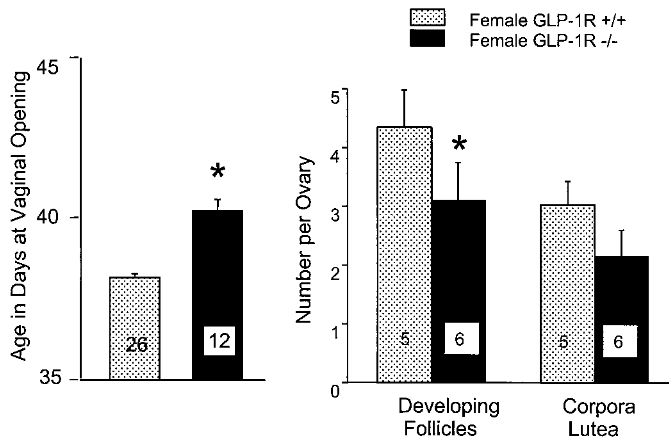

FIG. 3. Analysis of vaginal opening (puberty) and the number of developing follicles and corpora lutea in age-matched GLP-1R+/+ and GLP-1R−/− mice. *, $P < 0.05$, +/+ *vs.* −/− mice.

be less responsive than the wild-type controls. However, at the highest acoustic stimulus intensity (120 dB), this difference was reversed ($P < 0.01$; Fig. 7D). The heterozygous +/− mice exhibited responses midway between, but not significantly different from, those for the −/− and +/+ animals. To test whether the mild derangement in hypothalamic-pituitary-adrenal regulation, as denoted by decreased adrenal weights and heightened glucocorticoid responses to stress in GLP-1R−/− mice, might contribute to the observed differences in acoustic startle, the same groups of mice were retested after the addition of hydrocortisone to their drinking water for 6 days. Glucocorticoid replacement significantly diminished the difference between the genotypes in the startle response at high stimulus intensities to below the limits of statistical significance (Fig. 7D).

Discussion

The expression of the proglucagon gene in the CNS (7, 8) together with the detection of receptors for glucagon, GLP-1, and GLP-2 in anatomically distinct brain regions suggest that the PGDPs subserve multiple functions in the mammalian brain. The few studies examining the biology of PGDPs in the brain have focused on the hypothalamus and pituitary. Although the highest levels of proglucagon gene expression are localized to brain stem nuclei, proglucagon mRNA transcripts have also been detected in RNA isolated from rodent hypothalamus (7). Furthermore, primary cell cultures established from rat hypothalamus contain proglucagon mRNA transcripts and secrete the PGDPs in a regulated manner (28). The available data suggest that PGDPs such as GLP-1 may be either synthesized in the brain stem and transported to the hypothalamus or synthesized locally in specific hypothalamic nuclei (7, 29).

As GLP-1 is being tested as a potential therapeutic agent for the treatment of diabetes, and peripheral administration of GLP-1 modulates vasopressin and cortisol secretion *in vivo*, the potential neuroendocrine actions of GLP-1 may have relevance for understanding the consequences of GLP-1 administration *in vivo*. The findings that male GLP-1R−/− mice exhibit modest decreases in gonadal weight and female

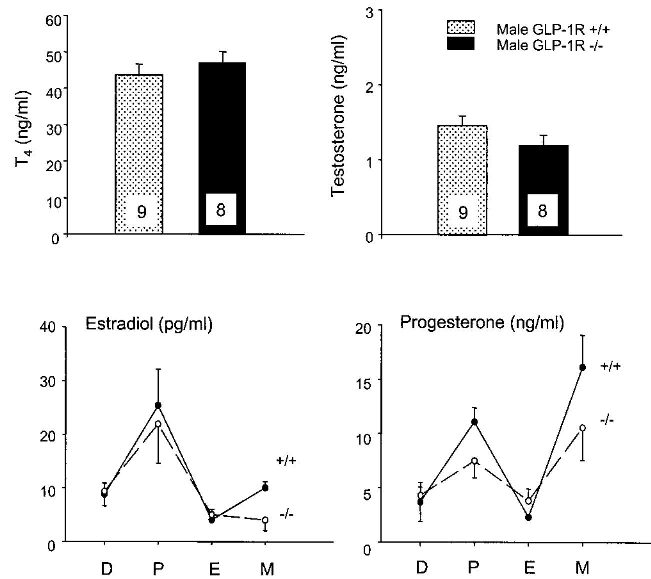

FIG. 4. *Upper panels*, Plasma levels of circulating T_4 and testosterone in adult (6-week-old) male mice. The numbers of observations are indicated on each bar. *Lower panels*, Estradiol and progesterone in GLP-1R+/+ and GLP-1R−/− mice. D, Diestrus; P, proestrus; E, estrus; M, metestrus. Hormone measurements were obtained in serum samples from 4–12 individual animals on each day of the cycle.

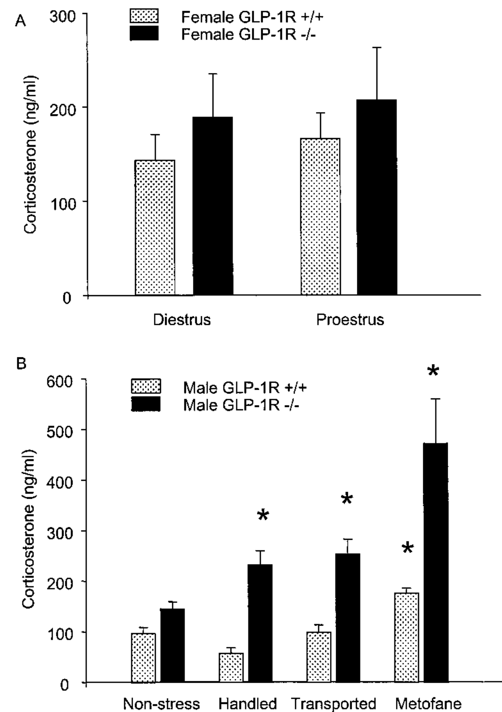

FIG. 5. A and B, Plasma corticosterone in female (A) and male (B) GLP-1R+/+ and GLP-1R−/− mice. The numbers of observations contributing to each mean are indicated in the diagram. Male mice were killed under four different conditions. 1) Nonstressed mice were removed from their home cages and immediately killed by asphyxiation in CO_2 gas. 2) Mice were transported in transfer cages to the necropsy laboratory, where they were killed 2–4 min later. 3) Mice were transported in their home cages up an elevator from the ground floor to a fourth floor laboratory, after which mice were removed from their cage and killed by CO_2 asphyxiation. 4) Mice were deeply anesthetized using metofane in a small 250-ml beaker, after which they were exsanguinated.

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mice exhibit a slight delay in the onset of puberty are consistent with a possible effect of GLP-1 on central regulation of the hypothalamic-pituitary-gonadal axis. Our previous findings demonstrated normal growth curves in adult male and female GLP-1R−/− mice, and studies of younger mice reported here demonstrate normal growth in GLP-1R−/− mice from birth to adulthood. Thus, the mild reproductive abnormalities observed in GLP-1R−/− mice cannot be ascribed, as previously suggested (17), to disturbances of energy homeostasis impacting on the reproductive axis. Furthermore, although pharmacological injection of icv GLP-1 inhibits feeding in rodents (12), GLP-1R−/− mice are not obese and do not exhibit short or long term disturbances in control of food intake or body weight (13, 16). Hence, it seems unlikely that reproductive abnormalities in GLP-1R−/− mice can be linked to dysregulation of nutrient intake.

Our analysis of reproductive function in GLP-1R−/− mice was motivated in part by the demonstration of GLP-1R on hypothalamic GT1 cells and the findings that icv GLP-1 stimulates LH secretion (17). These observations raised the possibility that GLP-1 may control pubertal development through a direct effect on hypothalamic GnRH secretion. We did not observe any overt changes in the reproductive function of GLP-1R−/− mice, and the number and distribution of anterior pituitary cell types, including corticotrophs and gonadotrophs, were normal in GLP-1R−/− mice. Although a consistent delay in the onset of the first ovarian cycle was found in the GLP-1R−/− animals, once cyclicity was established no differences between the −/− and wild-type +/+ mice were observed. The observation that GLP-1R−/− mice are fertile and do not exhibit impairment of reproductive capacity taken together with the normal circulating estradiol and progesterone levels clearly suggest that GLP-1 signaling is not essential for successful induction of ovulation, gonadal steroid production, or reproduction *in vivo*. In both +/+ and −/− mice, the majority of females exhibited regular 4-day cycles, which continued until the animals were at least 120 days of age. The slight reduction in circulating progesterone levels observed in the female GLP-1R−/− mice may reflect the marginal effect on the number of ovarian follicles observed at histological examination.

Although we did not specifically test sexual behavior, the fact that GLP-1R−/− mice breed normally suggests that loss of GLP-1 signaling does not result in a major reproductive behavioral deficit. In males, developmental masculinization apparently occurs normally, consistent with their normal growth curves and apparently normal reproductive performance in the breeding program. In studies not presented here, we found that sex differences in estrogen receptor distribution within the hypothalamus and preoptic area also develop normally in GLP-1R−/− mice; hence, loss of GLP-1 signaling does not interfere with sexual differentiation of the brain (Maclusky, N. J., J. Kim, and D. J. Drucker, unpublished observations).

Previous studies have yielded conflicting data on the interaction between GLP-1 and vasopressin in the rat CNS. The

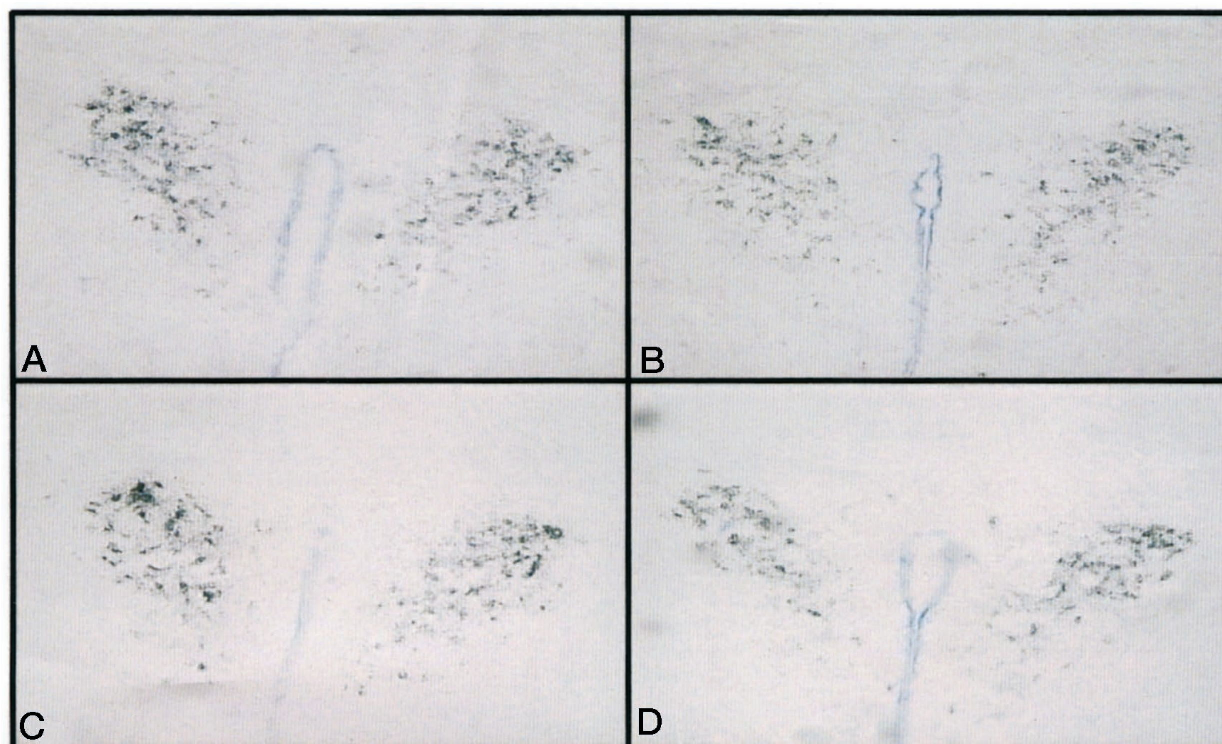

FIG. 6. Representative localization of CRH mRNA in the PVN of the mouse hypothalamus after *in situ* hybridization of coronal brain sections (25 μm) with a ^{35}S-labeled antisense riboprobe. A brightfield photograph of emulsion-coated autoradiograms revealed dense clusters of cells in the PVN. A, Fed wild-type control mice (*ad libitum* access to food and water); B, fed GLP-1R−/− mice (*ad libitum* access to food and water); C, 24-h fasted wild-type control mice (*ad libitum* access to water); D, 24-h fasted GLP-1R−/− mice (*ad libitum* access to water). Exposure time for A–D, 2 weeks. Magnification, ×100.

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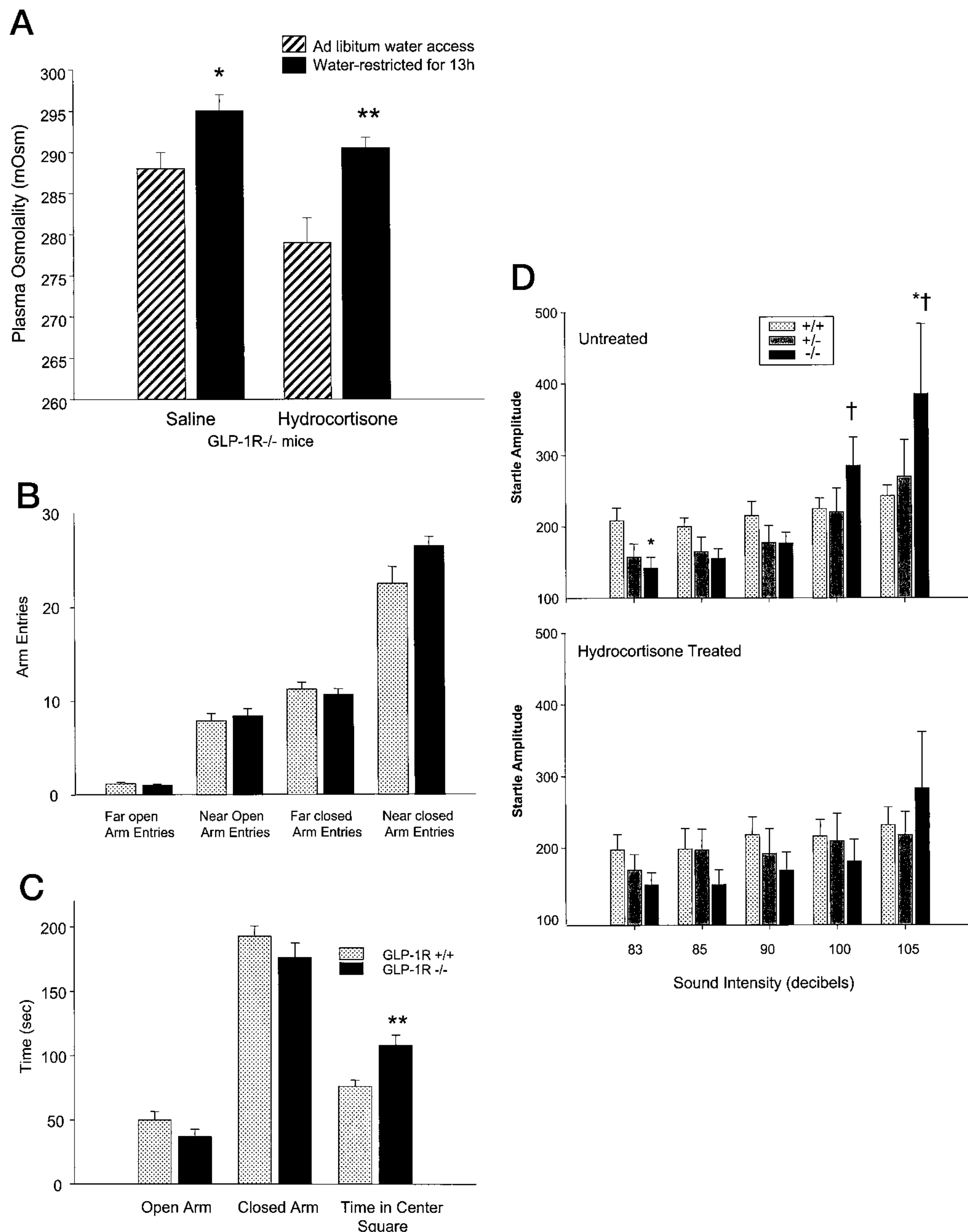

FIG. 7. A, Plasma osmolality of GLP-1R−/− mice in the basal and fluid-restricted state. Age-matched female GLP-1R+/+ (not shown) and GLP-1R−/− mice were analyzed before (*ad libitum* fluid intake) and after 13 h of fluid restriction. B, Behavior of GLP-1R−/− mice in the elevated plus maze. Mean entries for GLP-1R+/+ (n = 12) and GLP-1R−/− mice (n = 11) in different arms of the elevated plus maze are shown. C, Duration of arm entries of GLP-1R+/+ (n = 12) and GLP-1R−/− (n = 11) mice in the elevated plus maze. **, $P < 0.01$, +/+ *vs.* −/− mice. D, Acoustic startle responses in GLP-1R+/+ (n = 11), GLP-1R−/− (n = 10) and GLP-1R$^{+/-}$ heterozygous (n = 11) mice. *, $P < 0.05$, +/+ *vs.* −/− mice. †, $P < 0.05$ *vs.* results obtained in the same genotype at the 83-db stimulus intensity (Newman-Keuls *post-hoc* comparison). Acoustic startle responses were repeated (*lower panel*, hydrocortisone treated) in the same groups of mice after 6 days of treatment with hydrocortisone sodium succinate (0.5 mg/ml) in the drinking water.

finding of GLP-1R and vasopressin RNA colocalization in magnocellular neurons of the paraventricular and supraoptic nuclei (27) taken together with the induction of c-Fos immunoreactivity in the paraventricular nucleus (PVN) after icv GLP-1 (18) implies an anatomical and functional relationship between these two peptides. Nevertheless, icv injection of GLP-1 stimulated vasopressin secretion (18), whereas iv GLP-1 infusion inhibited vasopressin secretion (27). Our studies did not reveal any major impairment of osmoregulation in normal or fluid-restricted GLP-1R−/− mice before or after glucocorticoid treatment. Although these findings do not exclude the possibility that hypothalamic GLP-1R signaling in the PVN may contribute to physiological regulation of vasopressin release, GLP-1R signaling does not appear essential for sensing and/or responding to changes in plasma osmolarity *in vivo*.

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The finding that centrally administered GLP-1 activates c-*fos* activity in CRH hypothalamic neurons (18) raises the possibility that the GLP-1R may represent an upstream component of the systems regulating the CRH-ACTH-adrenal axis. The icv administration of GLP-1 increases corticosterone secretion in rats (18), and iv GLP-1 infusion increased both ACTH and plasma cortisol in human subjects (30), consistent with a role for GLP-1 in ACTH-dependent glucocorticoid secretion. Although disruption of GLP-1 signaling might conceivably affect development of the hypothalamic CRH^+ neurons and/or pituitary corticotrophs, thereby decreasing adrenal weight, these components of the HPA axis appear to develop normally in GLP-1R−/− mice. Furthermore, although adrenal weights are slightly lower in male and female GLP-1R−/− mice, levels of basal and stress-stimulated plasma corticosterone were clearly not reduced in the absence of GLP-1 signaling. In fact, stress-induced adrenal glucocorticoid secretion was paradoxically increased in GLP-1R−/− animals compared with that in wild-type controls killed under the same conditions.

The mechanisms underlying these subtle differences in corticosterone responses and adrenal weights remain unknown. Our initial hypothesis, based on findings that icv GLP-1 injections stimulated glucocorticoid release (18), suggested that abrogation of GLP-1 signaling might reduce basal and/or stress-induced corticosterone secretion. This hypothesis is obviously no longer tenable. Our data are consistent with the possibility that GLP-1 is involved physiologically in the regulation of CRH release; however, it remains unclear why disruption of GLP-1R signaling augments, rather than decreases, stress-activated adrenal corticosterone secretion. In wild-type animals, many of the systems that provide input to the PVN, including brain stem catecholaminergic neurons, the medial septum, and the hippocampus, also normally contain GLP-1R (31). The effects of GLP-1 on some of these centers could indirectly inhibit CRH release, resulting in enhanced stress responses in animals lacking GLP-1R. It is also possible that the lack of stimulatory input from GLP-1 to the PVN could have resulted developmentally in a compensatory disinhibition of CRH release in GLP-1R−/− mice. For example, GLP-1R−/− may exhibit a modest reduction in corticosterone feedback sensitivity, thereby normalizing basal CRH and circulating corticosterone levels. Under nonstress conditions, this would not result in any detectable phenotype. Under conditions of mild stress, however, such disinhibition might result in exaggerated CRH, and therefore corticosterone, release.

Despite the hypothesis that CRH expression may be mildly perturbed in GLP-1R−/− mice, we did not observe any significant difference in the expression of CRH mRNA between wild-type and GLP-1R−/− animals, with or without food restriction. Although the data from the plus maze and acoustic startle tests might be interpreted as consistent with an increase in intracerebral CRH release in GLP-1R−/− mice, they are inconclusive, as subtle abnormalities in multiple neuroendocrine circuits may ultimately influence the results of these test paradigms. In rats, icv CRH administration enhances acoustic startle responses (32). In both rats (33) and mice (34), icv CRH injections are anxiogenic in the elevated plus maze, reducing the number of entries into the open compared with the closed arms.

In the present study no significant differences were observed between +/+ and −/− mice in the relative numbers of entries into the open and closed arms of the plus maze. The only statistically significant effect was a reduction in the time spent in either the open or closed arms, with a concomitant increase in the time spent in the center square. This difference could indicate a general effect of the GLP-1R−/− phenotype on general locomotor or exploratory behavior, as opposed to a specific response. In the acoustic startle test, an increase in response with increasing sound stimulus intensity was observed in the GLP-1R−/− mice, consistent with an enhanced anxiogenic effect in these animals. However, differences between the GLP-1R−/− and GLP-1R+/+ mice were observed at both the lowest and the highest sound stimulus intensities, suggesting that the GLP-1R−/− mice not only respond differently to increased sound intensity, but also exhibit a different baseline level of activity in this test.

Whatever the underlying mechanisms, loss of GLP-1 function, as exemplified by targeted disruption of the gene encoding the GLP-1 receptor, clearly produces a subtle, but detectable, neuroendocrine phenotype, consistent with a putative physiological role for GLP-1 in the brain. Although GLP-1R−/− mice appear completely normal with respect to basal regulation of growth rates, thyroid, adrenal, and reproductive function, they do not respond normally to the challenge of a novel environment. The enhancement of corticosterone release in response to stress as well as the changes observed in the acoustic startle and elevated plus maze responses, all suggest that loss of GLP-1 signaling impairs CNS-pituitary-adrenal function. Furthermore, recent evidence suggests that systemic stress also activates GLP-1 neurons in the rat central nervous system, providing additional support for linkage between GLP-1 action and central regulation of the stress response *in vivo* (21). Under normal physiological conditions, therefore, GLP-1 may represent a modulatory component of the mechanisms regulating central and peripheral neuroendocrine and behavioral responses to stress.

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