GH in the dwarf dopaminergic D2 receptor knockout mouse: somatotrope population, GH release, and responsiveness to GH-releasing factors and somatostatin

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Abstract

Recently, the importance of the dopaminergic D2 receptor (D2R) subtype in normal body growth and neonatal GH secretion has been highlighted. Disruption of D2R alters the GHRH–GH–IGF-I axis and impairs body growth in adult male mice. The D2R knockout (KO) dwarf mouse has not been well characterized; we therefore sought to determine somatotrope function in the adult pituitary. Using immunohistochemistry and confocal microscopy, we found a significant decrease in the somatotrope population in pituitaries from KO mice ($P=0.043$), which was paralleled by a decreased GH output from pituitary cells cultured *in vitro*. In cells from adult mice the response amplitude to GHRH differed between genotypes (lower in KO), but this difference was less dramatic after taking into account the lower basal release and hormone content in the KO cells. Furthermore, there were no significant differences in cAMP generation in response to GHRH between genotypes. By Western blot, GHRH-receptor in pituitary membranes from KO mice was reduced to 46% of the level found in wildtype (WT) mice ($P=0.016$). Somatostatin induced a concentration-dependent decrease in GH and prolactin (PRL) secretion in both genotypes, and 1×10^{-7} M ghrelin released GH in cells from both genotypes ($P=0.017$) in a proportionate manner to

basal levels. These results suggest that KO somatotropes maintain a regulated secretory function. Finally, we tested the direct effect of dopamine on GH and PRL secretion in cells from both genotypes at 20 days and 6 months of life. As expected, we found that dopamine could reduce PRL levels at both ages in WT mice but not in KO mice, but there was no consistent effect of the neurotransmitter on GH release in either genotype at the ages studied. The present study demonstrates that in the adult male D2R KO mouse, there is a reduction in pituitary GH content and secretory activity. Our results point to an involvement of D2R signaling at the hypothalamic level as dopamine did not release GH acting at the pituitary level either in 1-month-old or adult mice. The similarity of the pituitary defect in the D2R KO mouse to that of GHRH-deficient models suggests a probable mechanism. A loss of dopamine signaling via hypothalamic D2Rs at a critical age causes the reduced release of GHRH from hypophyseotropic neurons leading to inadequate clonal expansion of the somatotrope population. Our data also reveal that somatotrope cell number is much more sensitive to changes in neonatal GHRH input than their capacity to develop properly regulated GH-secretory function.

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Introduction

The dopamine D2 receptor (D2R) is the predominant dopamine-receptor subtype in the anterior pituitary and mediates dopamine's inhibitory actions on lactotropes (Missale *et al.* 1998, Ben-Jonathan & Hnasko 2001, Diaz-Torga *et al.* 2002, Cristina *et al.* 2005). The importance of D2R in normal body growth and growth hormone (GH) secretion in the neonatal period has been highlighted

previously (Diaz-Torga *et al.* 2002). Our results indicate that lack of D2R alters the GH-releasing hormone (GHRH)–GH–insulin-like growth factor-I (IGF-I) axis, and impairs body growth in adult mice. An anatomical basis for an interaction of the dopaminergic and the GHRH system has been provided by colocalization studies, which indicate that a subset of GHRH neurons in the ventrolateral part of the arcuate nucleus contains tyrosine hydroxylase, the key enzyme of catecholamine biosynthesis in rats (Niimi *et al.* 1992, Zoli

et al. 1993), and mice (Phelps *et al.* 2003). Furthermore, dopamine neurons have perikarya that form ascending ventral and dorsal pathways to innervate limbic and hypothalamic structures, including the external layers of the median eminence, from where peptide hormones such as GHRH are released into the hypophysial portal circulation to be conveyed to the anterior pituitary.

Different models of dwarf mice and rats provide interesting information regarding the pathophysiology of growth retardation (Phelps & Hurley 1999). Two types of dwarf mouse, Snell (*du*, *dw^j*, or *Pit1*; Eicher & Beamer 1980) and Ames (*df* or *Prop-1*), with absent GH and prolactin (PRL) were used to elucidate physiological roles of GH and PRL long before the nature of the spontaneous mutations was known. The Snell dwarf mutations were later characterized as a base substitution and sequence rearrangement in the pituitary-specific transcription factor *Pit-1*, which is necessary for the expression of GH, PRL, and thyrotropin (TSH) (Li *et al.* 1990). The Ames dwarf mutation was localized to a novel homeobox-containing protein encoded on chromosome 11 (Sornson *et al.* 1996). This protein binds to DNA via the homeodomain in the promoter region of the *Pit-1* gene, and was named prophet of *Pit-1* or *Prop-1*. Therefore, null or hypomorphic mutations of the *Prop-1* gene also lead to a GH, PRL, and TSH-deficient phenotype. Another model of dwarf mice is the *lit/lit* (or GHRH-receptor (R)) mouse, in which there is a point mutation in the GHRH-R (Eicher & Beamer 1976, Lin *et al.* 1993).

In addition to spontaneous mutations, transgenic alterations of GH production have been described. For example, transgenic dwarf mice were produced by ablating somatotropes through the expression of the diphtheria toxin gene driven by the GH promoter (Behringer *et al.* 1988), or by a decrease in GHRH production induced by endogenous GH overexpression restricted to GHRH neurons (Flavell *et al.* 1996). On the other hand, the Laron dwarf mice, produced by targeted disruption of the GH receptor/GH-binding protein gene (GHR-knockout (KO) mice), are GH resistant (Zhou *et al.* 1997). As expected, each dwarf model presents unique characteristics and different alterations in the GHRH-GH-IGF-I axis, depending on the site of the primary defect.

The D2R KO dwarf mouse has not been completely characterized. Therefore, in the present study, we sought to determine if the reduction of serum GH concentration, which we have observed in the D2R KO mouse during the first month of life (Diaz-Torga *et al.* 2002), predicts impaired somatotrope cell number and function in the adult pituitary. We compared somatotrope cell number and *in vitro* responsiveness with GHRH, somatostatin (ST), and ghrelin in wildtype (WT) and KO male mice. Furthermore, we studied GHRH-R number in pituitaries, and determined if the pituitary D2R receptor was involved in the regulation of GH secretion in dispersed cells from young and adult mice.

Materials and Methods

Animals

Male D2 dopamine receptor KO mice (official strain designation B6; 129S2-*Drd2^{tm1low}* by the Induced Mutant Resource at The Jackson Laboratory, Bar Harbor, ME, USA), generated by targeted mutagenesis of the D2R gene in embryonic stem cells (Kelly *et al.* 1997, Asa *et al.* 1999), were used. The original F₂ hybrid strain (129S2/Sv X C57BL/6J) containing the mutated D2R allele was backcrossed for eight generations to WT C57BL/6J mice. Mutant and WT mice were generally the product of heterozygote crossings, and in all cases sibling controls were used. Mice were housed in groups of four or five with mixed genotypes in an air-conditioned room with lights on at 0700 h and off at 1900 h. They had free access to laboratory chow and tap water. WT, heterozygous, and KO mice were identified by PCR of genomic DNA, as previously described (Diaz-Torga *et al.* 2002). Animals were used at 6–8 months. All experimental procedures were reviewed and approved by the institutional animal care and use committee of the Instituto de Biología y Medicina Experimental, Buenos Aires (Division of Animal Welfare, Office for Protection of Research Risks, National Institutes of Health, A#5072-01).

Drugs

Unless specified, all chemicals were purchased from Sigma.

Immunohistochemistry and confocal laser microscopy

Immunostaining was performed on paraffin-embedded sections of 8-month-old WT and D2R KO mice of C57BL/6 congenic strains. We used rabbit polyclonal antibody against mouse GH (dilution 1:1000, NHPP, NIDDK-AFP-5672099). After rinsing in PBS, the sections were incubated at room temperature for 2 h with fluorescein isothiocyanate (FITC) goat anti-rabbit immunoglobulin G (dilution 1:100; Zymed Laboratories, Inc., San Francisco, CA, USA). After rinsing in PBS, the sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) to prevent fading of the immunofluorescence reaction. Sections were examined on a C1 Plan Apo 60×/1.4 oil confocal laser-scanning system (Nikon, Tokyo, Japan). The excitation wavelength was 488 nm for FITC. Specificity studies, carried out by omitting primary antisera or pre-absorbing the primary antisera with homologous antigen excess, all showed the absence of the fluorescent signal. Four animals were used for each experimental group, and a minimum of four to five pituitary sections obtained at different levels of each gland were used for quantitation. The number of GH positive cells was expressed as a percentage of total nucleated cells in the section. Nucleated cells were evidenced by counterstaining with propidium iodide. For clarity of the figure, we include only the image visualized in green fluorescence (488 nm).

Cell dispersion and culture

Anterior pituitaries from male WT and KO mice were weighed and dissociated into single cells. They were placed in chambers containing freshly prepared Krebs–Ringer bicarbonate buffer without Ca^{2+} or Mg^{2+} . The buffer contained 14 mM glucose, 1% BSA, 2% minimum essential medium (MEM) amino acids, 1% MEM vitamins (Life Technologies, Inc.), and 2 mM glutamine and was previously gassed for 15 min with 95% O_2 /5% CO_2 , and adjusted to pH 7.35–7.40. Buffer was filtered through a 0.45 μm pore diameter membrane (Nalgene, Nalge Nunc International, Rochester, NY, USA). Pituitaries were washed three times with Krebs–Ringer bicarbonate buffer and then cut into 1 mm pieces. Fragments obtained were washed and incubated in the same buffer containing 0.5% trypsin for 30 min at 37 °C in 95% air/5% CO_2 , followed by an additional 2 min with 50 μl DNase I (1 mg/ml; Worthington Biochemical Corp., Lakewood, NJ, USA). Digestion was ended by adding an excess of fetal bovine serum (FBS). Fragments were dissociated to single cells by gentle trituration through Pasteur pipettes. The resulting suspension was filtered through nylon gauze (160 μm pore size) and centrifuged for 10 min at 1000 g . Before centrifugation, an aliquot of cellular suspension was taken to quantify pituitary cell yield, using a Neubauer chamber. Viability of cells, determined by Trypan blue exclusion, was always greater than 90%. Cells (35 000 cells/well) were cultured for 5 days in Dulbecco's modified Eagle's medium, 10% horse serum, and 2.5% FBS. Cells were then washed and stimulated with different concentrations of human GHRH (hGHRH)–(1–29) amide (provided by the National Institute of Diabetes and Digestive and Kidney Diseases's (NIDDK's) National Hormone and Peptide Program and Dr A F Parlow), somatostatin (Somatostatin-UCB; Rontag, Buenos Aires, Argentina), ghrelin (Peptide Institute, Inc., Osaka, Japan), and dopamine (3,4-hydroxyphenethylamine hydrochloride) for 24 h in Dulbecco's modified eagle medium +F-12 nutrient mixture + NaHCO_3 (BIC) 0.5% BSA, 0.1 mM ascorbic acid medium, without serum. For GHRH, proportional results were obtained after 1-h of incubation (not shown). Cell culture was performed as described previously (Gonzalez Iglesias *et al.* 2000).

RIAs

PRL and GH were measured by RIA using kits provided by the NIDDK (Dr A F Parlow, National Hormone and Pituitary Program (NHPP), Torrance, CA, USA). Assays were performed using the appropriate quantity of diluted medium from cultured cells. Results are expressed in terms of mouse PRL standard RP3 or mouse GH standard AFP-10783B. Intra- and interassay coefficients of variation were 7.2 and 12.8%, and 8.4 and 13.2%, for PRL and GH respectively.

Western blot

Anterior pituitaries were homogenized in 80 μl ice-cold homogenization buffer containing 10 mM PO_4Na_2 , 10 mM MgCl_2 and a mix of protease inhibitors in a handheld micro-tissue homogenizer. The homogenate was centrifuged at 800 g for 5 min at 4 °C to remove unbroken cells and nuclei. Membranes were pelleted at 20 000 g for 30 min at 4 °C and resuspended in homogenization buffer containing 5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) on ice for 90 min at 4 °C. An aliquot of the detergent lysates was taken to quantify proteins by the Lowry method. Thirty micrograms of proteins in 10 μl lysate were mixed with 10 μl 2 $\times$ sample buffer (60 mM Tris-HCl, 4% SDS, 20% glycerol, 0.02% bromophenol blue, and 50 mM dithiothreitol, pH 6.8) and subjected to 12% SDS-PAGE. The gel was then blotted onto a nitrocellulose membrane and probed with the corresponding primary antibody followed by a secondary antibody conjugated with horseradish peroxidase. Polyclonal rabbit receptor GHRH antibody (1:1000) was used, and specificity was described in detail in Gaylinn *et al.* (1999). Consistent with previous GHRH cross-linking studies, a band of approximately 52 kDa was detected as well as other unspecific bands, but after treatment with peptide N-Glycosidase F (PNGase F), according to the manufacturer's protocols (New England Biolabs), only the receptor-specific band shifted to a greater apparent mobility, as expected from our previous observations of receptor glycosylation (Gaylinn *et al.* 1994). Identification of this 52 kDa glycoprotein band as GHRH-R was shown previously by inclusion of excess synthetic peptide GHRH-R immunogen, which blocked the labeling of the specific GHRH-R band, by inclusion of a sample with preimmune rabbit serum, which yielded no band, and by photoaffinity cross-linking with GHRH (Gaylinn *et al.* 1999). Monoclonal mouse actin antibody (Ab-1) was purchased from Labvision Co. (Freemont, CA, USA). Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham). For repeated immunoblotting, membranes were incubated in stripping buffer (62.5 mM Tris, 2% SDS, and 100 mM mercaptoethanol; pH 6.7) for 40 min at 50 °C and reprobed. Band intensities were quantified using the ImageQuant software (version 5.2, Molecular Dynamics), and the intensity of the GHRH-R band was normalized to the intensity of the respective actin band.

Intracellular cAMP measurement

For intracellular cAMP measurements, cells obtained as described earlier were plated in 24-well culture plates. After 5 days of culture, pituitary cells (250 000 cells/well) were washed and pre-incubated with BIC-BSA medium for 2 h. The cells were then incubated with hGHRH (1×10^{-7} to 1×10^{-9} M) or buffer for 30 min in the presence of 3-isobutyl-1-methylxanthine (0.1 mM). After incubation, cells were placed on ice and washed with BIC-BSA, 0.7 ml cold ethanol was added to each well, cells were scraped,

transferred to tubes, sonicated for 15 s, heated for 5 min at 95 °C, and centrifuged for 5 min at 9400 g. Supernatants were evaporated, and the dry extracts were suspended in cAMP RIA buffer (NaAc 50 mM, pH 6.2). Intracellular cAMP was measured by RIA as described previously (Del Punta *et al.* 1996) using the antibody provided by the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDKD). Assay sensitivity was 3.3 pg (10 fmol)/tube. Intra- and interassay coefficients of variation were 8.1 and 10.5% respectively. Experiments were repeated four times. Results are expressed as percentage increase over basal levels (100%).

Statistical analyses

Results are expressed as means $\pm$ S.E.M. *In vitro* culture experiments were analyzed by two-way ANOVA for independent measures for the effects of genotype and drug treatment (hGHRH, ST, ghrelin, or dopamine). In all cases, if the *F* interaction was found to be significant, then individual means were compared by Tukey's honest significant difference; if it was not significant, then the groups of means were analyzed by the same tests. Results of GHRH-R expression, and number of somatotropes were analyzed by *t*-test. $P < 0.05$ was considered significant.

Results

GH release from pituitary cells cultured in vitro

Basal GH release (ng/ml) was similar in pituitary cells from 20-day-old mice from both genotypes cultured *in vitro* (Fig. 1A); but in 6–8-month-old mice, basal GH release was lower in cells from male KO mice than in cells obtained from WT animals ($P = 0.0027$; Fig. 1B, control levels). In accordance, using confocal microscopy we found a significant decrease in somatotrope population in pituitaries from adult D2R KO mice (Fig. 2). In WT pituitaries somatotropes represented $40.0 \pm 3.7\%$, and in KO $25.7 \pm 0.5\%$, of total cells

($P = 0.043$). No differences in total gland weight were evidenced as described previously (Diaz-Torga *et al.* 2002).

To assess the functional capacity of the somatotrope populations, pituitary cells from KO and WT mice were challenged with GHRH (1×10^{-7} to 1×10^{-10} M). In cells from 6- to 8-month-old mice, the response amplitude to GHRH differed between genotypes, but this difference was less dramatic after taking into account the lower basal release and hormone contents in the KO cells. A two-factor ANOVA indicated that GH levels were significantly lower in KO cells compared with WT (*F* genotype (1,16) = 43.48; $P = 0.0027$) and there was no significant interaction between genotype and GHRH concentration (*F* interaction (1,16) = 0.76; $P = 0.56$, Fig. 1B). GHRH released GH at concentrations of 1×10^{-7} to 1×10^{-8} M (Fig. 1B, $P = 0.00047$ and 0.0045 respectively versus control) in both genotypes. The effective concentration 50 (EC50) was 2.16 and 1.85×10^{-9} M for WT and KO respectively (not significant). Even though GHRH sensitivity was similar between genotypes, the absolute GH release induced by GHRH was significantly lower in cells from KO mice compared with those from WT mice. In contrast, in 20-day-old mice GHRH-induced GH release was similar between genotypes in terms of both sensitivity and amplitude (Fig. 1A; *F* genotype (1,47) = 1.39, $P = 0.24$; and $P = 0.00032$ and 0.026 control versus 10^{-7} and 10^{-8} M GHRH respectively for both genotypes). PRL release was unaffected by GHRH exposure (data not shown).

GHRH-induced intracellular cAMP production in anterior pituitary cells in vitro

Figure 3 compares cAMP levels in extracts from adult male WT and KO pituitary cell cultures exposed to GHRH (1×10^{-7} to 1×10^{-9} M) for 30 min. GHRH increased cAMP generation at the concentration of 1×10^{-7} M ($P = 0.00017$), and no differences were observed between genotypes (interaction between genotype and treatment was not significant, *F*(1,47) = 0.32; $P = 0.81$). The EC50 for

Figure 1 Effect of growth hormone-releasing hormone (GHRH) on growth hormone (GH) release in dispersed pituitary cells from (A) 20-day-old and (B) adult male mice of both genotypes. WT, wildtype; KO, D2R knockout mice. * $P < 0.05$ versus respective control. $n = 5$. GHRH8:GHRH (1×10^{-8} M).

Figure 2 Immunofluorescent staining combined with confocal laser microscopy in the pituitary of adult wild type (WT) and D2R knockout (KO) mice. Each image is the representative of staining patterns seen in specimens from four independently examined mice. Growth hormone is visualized in the green immunofluorescent channel. Objective lens magnification $\times 40$.

GHRH-induced cAMP generation was 1.14 and 1.81×10^{-8} M for WT and KO respectively (not significant).

GHRH-R expression in pituitaries from WT and KO male mice

GHRH-R expression was evaluated by Western blot of CHAPS-extracted crude pituitary membranes. Consistent with previous GHRH cross-linking studies, a band of approximately 52 kDa was detected and after treatment with PNGase F, the receptor-specific band was shifted to a greater apparent mobility, as expected from our previous observations of receptor glycosylation (Gaylinn *et al.* 1994). GHRH-R protein in pituitary membranes from KO mice was reduced to 46% of the level found in WT mice (Fig. 4, $P=0.016$).

Figure 3 Effect of growth hormone-releasing hormone (GHRH) (GHRH7:GHRH (1×10^{-7} M)) on intracellular cAMP production expressed as percent variation in basal levels. * $P<0.05$ versus respective control. $n=4$.

Somatostatin-induced inhibition of GH and PRL secretion in anterior pituitary cells in vitro

Basal GH levels were lower and basal PRL levels were higher in secreted media from KO compared with WT cells ($P=0.00062$ and $P<0.00001$ for GH and PRL respectively).

Figure 4 Pituitary growth hormone-releasing hormone receptor (GHRH-R) content evaluated by Western blot and normalized to the respective actin content of the sample in wild type (WT) and knockout (KO) male mice; * $P<0.05$ versus WT. $n=8, 9$. Below are the representative bands and biotinylated weight markers corresponding to 60, 50, and 40 kDa on the left of the GHRH-R bands.

ST induced a concentration-dependent decrease in GH and PRL secretion in both genotypes (Fig. 5A and C; for GH: $P=0.00016$, 0.0014 , and 0.025 ; for PRL $P=0.00017$, 0.0039 , and 0.066 for the concentrations of 10^{-7} , 10^{-8} , and 10^{-9} M versus control respectively). Interaction (genotype $\times$ drug) was not significant, indicating a similar sensitivity to ST (for GH: $F_{\text{interaction}}(1,36)=0.80$, $P=0.77$; for PRL $F_{\text{interaction}}(1,42)=1.57$, $P=0.21$). Furthermore, the ST-induced decrease in the GH-releasing effect of 10^{-8} GHRH was also similar between genotypes (Fig. 5B, $P=0.00012$, 0.00013 , and 0.0018 for ST 10^{-7} , 10^{-8} , and 10^{-9} combined with GHRH, versus GHRH only respectively).

Effect of ghrelin on GH release

Ghrelin (1×10^{-7} M) released GH in cells from both genotypes (Fig. 6A, $P=0.017$). The magnitude of increase was smaller than that induced by GHRH (see Fig. 1). ST decreased the ghrelin (1×10^{-7} M)-induced GH release similarly in both genotypes (Fig. 6B, $P=0.029$; ghrelin 10^{-7} M versus ghrelin 10^{-7} M + somatostatin 10^{-8} M). Ghrelin did not modify PRL release (not shown).

Effect of dopamine on GH and prolactin release from pituitary cells

Finally, we tested if there was a direct effect of dopamine on GH secretion in cells from both genotypes at 20 days and 6 months of life. As expected, we found that dopamine could reduce PRL levels at both ages in WT mice and not in KO mice (Fig. 7A and B), but there was no consistent effect of the neurotransmitter on GH release in either genotype at the ages studied (Fig. 7C and D).

Discussion

In a previous work, we demonstrated that the D2R KO male mouse is growth retarded (Diaz-Torga *et al.* 2002).

The present study demonstrates that in the adult male D2R KO mouse, there is a reduction in pituitary GH release related to a decrease in somatotrope population size, and a decrease of the gland GH secretory activity. Nevertheless, these mice have apparently normal serum GH levels, which suggests that different mechanisms compensate for the decreased somatotrope population or that subtle alterations in GH pulsatility occur *in vivo*.

What are the contributing factors leading to the diminished expansion of the somatotrope population? In a previous work, we observed that in D2R KO mice serum GH levels were markedly decreased during the first month of life, and in this period D2R antagonists could release GH in WT mice (Diaz-Torga *et al.* 2002), suggesting that D2Rs are necessary for the correct GHRH-GH function in the neonatal period. In the present work, we point to an involvement of D2R signaling at the hypothalamic level as dopamine did not modify GH acting at the pituitary level either in 1-month-old or adult mice. It has been documented that dopamine D1Rs and not D2Rs participate in GH release at the pituitary level (Bluet-Pajot *et al.* 1990). Hypothalamic interaction of the dopaminergic and GHRH system has been provided by colocalization studies in rats and mice of GHRH and tyrosine hydroxylase neurons in the arcuate nucleus (Niimi *et al.* 1992, Zoli *et al.* 1993, Phelps *et al.* 2003). Furthermore, it has been shown that catecholamine-containing neurons innervate GHRH neurons in the rat arcuate nucleus (Sato *et al.* 1989).

Reduced levels of GHRH within the hypothalamus or GHRH action in the pituitary at a critical developmental window have a long lasting impact on body weight (Lin *et al.* 1993, Robinson *et al.* 1993, Cella *et al.* 1994) and induce an inadequate clonal expansion of the somatotrope population. The requirement of GHRH for the normal development of the somatotrope lineage is evident from studies examining the etiology of growth retardation in the spontaneous mutant mouse, *lit/lit*, with a point mutation in the GHRH-R gene in which somatotropes fail to proliferate normally, resulting in a mature pituitary containing a limited number of GH cells (Lin *et al.* 1993). Likewise, an experimentally induced reduction of

Figure 5 Effect of somatostatin (ST) on (A) growth hormone (GH) release and (C) prolactin (PRL) release in dispersed pituitary cells from adult male mice of both genotypes. WT, wildtype; KO, D2R knockout mice; ST7, ST (1×10^{-7} M). (B) Effect of ST (1×10^{-8} M) on growth hormone-releasing hormone (GHRH) (1×10^{-8} M)-induced GH release. * $P < 0.05$ versus respective control in A, B, and C, and $P < 0.05$ versus GHRH8 in B, $n = 6$.

Figure 6 (A) Effect of ghrelin (ghre) on growth hormone (GH) release in dispersed pituitary cells from adult male mice of both genotypes. ghre7, ghrelin (1×10^{-7} M). (B) Effect of somatostatin (1×10^{-8} M) on ghrelin (1×10^{-7} M)-induced GH release. * $P < 0.05$ versus respective control; ^a $P < 0.05$ versus ghre7, $n = 4$.

GHRH during the fetal period, by passive immunization with GHRH antiserum, results in growth retardation characterized by a decreased pituitary size and GH content (Cella *et al.* 1994). In this respect, it has been shown that GHRH plays a major stimulatory role in the regulation of the Pit-1 mRNA

concentration (Müller *et al.* 1999), and in the direct regulation of pituitary GHRH-R mRNA and the GH gene.

The similarity of the pituitary defect in the D2R KO mouse compared with that of the earlier GHRH-deficient models suggest that the reduced release of GHRH within the

Figure 7 Effect of dopamine (DA 1×10^{-6} – 1×10^{-8} M) on prolactin (PRL) (ng/ml A and B) and growth hormone (GH) (ng/ml C and D) release in dispersed pituitary cells from 20-day-old (A and C) and adult (B and D) male mice of both genotypes. * $P < 0.05$ versus respective control. $n = 5$. DA7:DA (1×10^{-7} M).

hypothalamus related to the lack of D2Rs at a critical age produces inadequate clonal expansion of the somatotrope population.

The GHRH-R is a member of the G-protein-coupled receptor superfamily, using cAMP as its predominant second messenger. In the present report, we have examined GH secretion and cAMP production in response to GHRH to search for a defect in the GHRH signal transduction pathway that may be linked to GH deficiency. GHRH-induced GH release and cAMP generation in KO mice were reduced but in general, proportional to the low pituitary GH cell number. Nevertheless, total GH response per pituitary was reduced, and this could account for lower IGF-I and IGF-binding protein-3 observed in adult KO mice (Diaz-Torga *et al.* 2002).

The present study demonstrates that the reduction in GH levels previously observed in the D2R KO mouse is due to a decrease in somatotrope population size. Because GHRH not only stimulates somatotrope proliferation, but also plays an obligatory role in the maintenance of GH biosynthesis and release, we were surprised to find that the reduction in somatotrope cell number observed did not impair the functional capacity of somatotropes. This suggests that the mitotic capacity of somatotropes is very sensitive to alteration in neonatal GHRH secretion, while the maintenance of the GH biosynthetic and secretory processes has less sensitivity to such changes.

Similarly, in the tyrosine hydroxylase-human GH (TH-hGH) transgenic dwarf mouse in which hypothalamic expression of the hGH gene decreases GHRH (Kineman *et al.* 1996), pituitaries contain about one-half of the normal complement of GH-secreting cells compared with WT pituitaries, but the amount of GH released per somatotrope under both basal and stimulated conditions is indistinguishable between the two groups, even though the amount of GH released by pituitary is lower (Kineman *et al.* 1996). In contrast, in other models of dwarfism, such as Snell (*dw*), Snell-Jackson (*dsj*) or Ames and the *dr* rat, somatotropes are almost unresponsive to GHRH. Furthermore, in the dwarf (*dw/dw*) rat, which has a severe GH deficiency, GHRH failed to increase cAMP indicating an intrinsic deficit of GHRH signal transduction pathway of somatotropes (Tierney & Robinson 2002).

The decrease that we found in the concentration of pituitary GHRH-Rs may also account for the decrease in GHRH action. As mentioned earlier, endogenous neonatal GHRH may be important for normal expression of GHRH-Rs. For example, it has been described that in the spontaneous dwarf rat there is an enhanced GHRH release due to a deficient negative feedback produced by a point mutation of the GH-receptor gene, which correlates with an increase in pituitary GHRH-R mRNA (Kamegai *et al.* 1998). Conversely, the decrease in hypothalamic GHRH in the transgenic dwarf mouse expressing the TH-hGH fusion gene in the hypothalamus is associated with a major reduction in pituitary GHRH-R mRNA (Szabo *et al.* 1995).

We also sought to determine whether there were differences between genotypes in GH sensitivity to another releasing

factor, ghrelin, which acts through a different signaling cascade within the pituitary. Ghrelin, a peptide predominantly produced by the stomach, has been discovered as a natural ligand of the GH secretagog receptor type 1a (Smith 2005). Its signal transduction involves activation of phospholipase C (via G protein), generation of inositol phosphate and diacyl glycerol, and increased intracellular Ca^{2+} . It has been described that ghrelin-induced GH release is inhibited in mice with dwarfism due to the loss of $\text{G}_{q/11}$ family G proteins in the central nervous system (Wettschureck *et al.* 2005). However, we found no significant differences in ghrelin-induced GH release between genotypes, other than the proportional decrease due to diminished number of somatotropes. Furthermore, PRL secretion is elicited by the ghrelin mimetic, GHRP-6 in the *dw/dw* phenotype (Carmignac *et al.* 1998), but not in our dwarf model.

D2Rs and the ST receptor SSTR5 interact physically through hetero-oligomerization in neurons to create a novel receptor with enhanced functional activity (Rocheville *et al.* 2000). Nevertheless, no differences were found in ST control of basal and GHRH- or ghrelin-stimulated GH release. Furthermore, it was interesting to determine that there was no increase in ST-induced PRL reduction to compensate for the lack of dopamine inhibition.

The results of our studies provide evidence for a defect in somatotrope population and pituitary GHRH-R expression, which limits the overall GH-secretory capacity of the gland in the D2R KO mouse. Low GH secretory capacity may be found in the majority of children with short stature due to known hypothalamic dysfunction who respond to acute stimulation with GHRH though generally in a subnormal manner, and in whom growth response to long-term treatment is frequently limited (Schriock *et al.* 1984).

The results presented herein using the D2R-deficient mouse support the involvement of the D2R in growth regulation, and point to a permanent alteration in somatotrope population in this model.

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