Increased lactotrophs despite decreased somatotrophs in the dwarf (*dw/dw*) rat: a defect in the regulation of lactotroph/somatotroph cell fate?

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Abstract

The dwarf (*dw/dw*) rat differs from all other rodent models of GH deficiency in that its pituitary prolactin (PRL) content is normal or even increased. We have now studied this throughout postnatal development, using a combination of immunocytochemistry, RIA and fluorescence-activated cell sorting (FACS) and analysis. Compared with normal Albino Swiss (AS) rats, adult *dw/dw* rats showed a profound reduction in pituitary GH content accompanied by increased PRL content, significantly so in females (AS vs *dw/dw*; $P < 0.01$). Somatotroph hypoplasia was evident in the adult *dw/dw* rats, with most GH⁺ cells showing weak immunostaining, whereas many more strongly stained PRL cells were evident in pituitary sections from *dw/dw* rats. FACS analysis confirmed both somatotroph hypoplasia and relative lactotroph hyperplasia in *dw/dw* rats at all ages studied (9–144 days); the difference in somatotrophs increased with age whereas the difference in lactotrophs declined with age. At 9 days, the percentage of lactotrophs was 10-fold higher in *dw/dw* rats than in AS rats. Young *dw/dw* rats also had a higher proportion of mammosomatotrophs than AS rats, although this

difference disappeared as the mammosomatotroph proportions increased with age in both strains. GHRH released GH from both *dw/dw* and AS cells maintained in culture for 5 days. The sensitivity to GHRH and the amount of GH released was lower in the *dw/dw* cultures, mostly explained by their fewer GH cells and lower initial GH content. GHRH increased cAMP in AS but not in *dw/dw* cultures, even when these were greatly enriched for *dw/dw* somatotrophs by FACS sorting prior to culture. These results suggest that GHRH-induced cAMP stimulation is required for trophic effects on GH synthesis and somatotroph proliferation, but is not required for GHRH-stimulated GH release. The inverse changes in somatotroph and lactotroph numbers suggest that the *dw/dw* mutation disturbs the mechanism that specifies and retains appropriate numbers of somatotrophs in their differentiated state, and results in a higher proportion of the remaining cells progressing to lactotrophs. The *dw/dw* phenotype is thus not confined to somatotrophs.

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Introduction

Much information on the physiology and pathophysiology of growth hormone (GH) has come from the study of dwarfism in mice, and has led to the discovery of genes involved in the prenatal development of somatotrophs (Li *et al.* 1990, Sornson *et al.* 1996), or the postnatal regulation of GH synthesis and release (Lin *et al.* 1993), all of which have subsequently been recognised to underlie equivalent human pituitary pathologies (Haugen & Ridgeway 1995, Cohen *et al.* 1996, Wu *et al.* 1998, Salvatori *et al.* 2001). Two genetic models of dwarfism have also been described in the rat. One, the spontaneous dwarf (*dr/dr*) rat, carries a mutation in its GH gene and cannot produce an active GH protein (Takeuchi *et al.* 1990). The other is the dwarf

(*dw/dw*) rat, first identified by us in 1988 (Charlton *et al.* 1988).

Although the genetic basis of the *dw/dw* mutation has not yet been identified, it is an autosomal recessive disorder, causing a severe but sub-total GH deficiency. These *dw/dw* rats secrete GH in the typical sexually dimorphic rodent pulsatile pattern, albeit with much lower peak amplitudes (Legraverend *et al.* 1992), and respond acutely to GH secretagogues such as GH-releasing hormone (GHRH) or growth hormone releasing peptide-6 (GHRP-6) with a GH secretory response, reduced in proportion to their pituitary content (Charlton *et al.* 1988, Carmignac & Robinson 1990).

GHRP-6 also induces prolactin (PRL) release, but when this was examined in *dw/dw* female rats,

unexpectedly large PRL responses to GHRP-6 were observed (Carmignac *et al.* 1998, Thomas *et al.* 1999). Subsequent measurements showed a significantly higher pituitary PRL content in adult *dw/dw* animals, although their basal plasma PRL levels were normal (Carmignac *et al.* 1998, Thomas *et al.* 1999). Previous studies had reported normal, or only mildly elevated, PRL content in *dw/dw* rats at different ages (Charlton *et al.* 1988, Bartlett *et al.* 1990). In most other GH-deficient rodent dwarf models, PRL content is reduced (for review see Phelps 1994), often profoundly so (Li *et al.* 1990, Sornson *et al.* 1996). An increase in PRL accompanying GH deficiency in *dw/dw* rats is thus highly unusual, but the cellular basis of this is unknown.

To address this, we have now characterised the somatotroph, lactotroph and mammosomatotroph populations in this *dw/dw* strain, using a combination of immunocytochemistry, fluorescence-activated cell sorting (FACS) and *in vitro* cell culture. We show that, in parallel with somatotroph hypoplasia, the *dw/dw* mutation causes an early and sustained increase in lactotroph numbers and PRL storage, but no overall change in the mammosomatotroph population. We confirm that the cAMP response to GHRH is undetectable, not only in mixed pituitary cell cultures from *dw/dw* rats (Downs & Frohman 1991), but even in cultures of *dw/dw* somatotrophs highly enriched by FACS sorting, and that the GH release continues in the absence of a cAMP response to GHRH. Some of these data have been reported in preliminary form (Gilbert & Robinson 1998).

Materials and Methods

Animals

The *dw/dw* rats were from the colony originally described by us (Charlton *et al.* 1988) and maintained on an Albino Swiss (AS) background at the National Institute for Medical Research ever since. Normal AS rats were used as controls. All rats were housed in plastic cages with up to six animals per cage in a dedicated animal facility. Animals were allowed free access to water and standard laboratory rat chow, under a 12 h light:12 h darkness regime, at constant temperature, and maintained under UK Home Office guidelines.

Immunocytochemistry

Whole pituitaries from 10-week-old male and female AS and *dw/dw* rats ($n=3$) were fixed overnight in 4% paraformaldehyde after which paraffin-embedded 4 μ m sections were mounted on poly-L-lysine-coated slides. Cells grown on cover slips in tissue culture were fixed for 30 min in 4% paraformaldehyde. Cells and de-waxed sections were incubated in 0.9% H₂O₂ in methanol for

30 min, washed and incubated in 0.1% trypsin (15 min, 37 °C), washed in 0.05% Triton, and then in Tris-buffered saline (TBS: 0.05 M Tris-HCl pH 7.6, 137 mM NaCl). After blocking in NS/BSA/TBS (5% BSA + 20% normal serum (NS) of the same species as the secondary antibody, in TBS) for 30 min, they were incubated with primary antibody overnight at 4 °C or for 1 h at room temperature. They were washed and then incubated for 30 min in secondary antibody in NS/BSA/TBS at room temperature. The antibody systems were (i) monkey anti-rat (r) GH (1:2000; National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK), Bethesda, MD, USA) followed by biotinylated goat anti-human-IgG (1:200; Vector Laboratories, Burlingame, CA, USA) or (ii) rabbit anti-rPRL (1:8000; NIDDK) followed by biotinylated swine anti-rabbit-IgG (1:200; Dako, Ely, Cambs, UK). GH⁺ve cells were stained using diaminobenzidine tetrahydrochloride (Sigma) after incubation with peroxidase-conjugated avidin-biotin complex (Dako). PRL⁺ve cells were stained with Fast Red (Dako) after incubation with alkaline phosphatase-conjugated avidin-biotin complex (Dako). Sections were counterstained with haematoxylin (BDH, Lutterworth, Leics, UK). The staining specificity of these reagents has been previously established (Flavell *et al.* 1996).

Cells immunostained for GH and PRL were counted by direct microscopy. For each animal, 1000–2000 cells were counted over three different areas, using an eyepiece graticule at a magnification of $\times 40$, to estimate the proportion of the total cell number in each animal ($n=3$ per group). The results for each cell type were then expressed as a percentage (mean $\pm$ S.E.M.).

Primary culture of anterior pituitary cells

Anterior pituitaries from 10-week-old AS and *dw/dw* rats were enzymically dispersed using modifications of previously described methods (Weiner *et al.* 1983, Ceda *et al.* 1987). Briefly, pituitaries were chopped into small pieces and dispersed in Hanks' Balanced Salts Solution (HBSS) (Sigma) containing 0.3 mg/ml collagenase type 1A (Sigma) and 0.25% trypsin (Sigma) at 37 °C. After 30 min, DNase 1 (Sigma) was added (final concentration 0.05 mg/ml), the cells were triturated occasionally for 2–3 h and then passed through a 40 μ m cell strainer. Dispersed cells were >98% viable as assessed by Trypan Blue exclusion. The suspension was centrifuged at 130 g for 10 min, the pellet washed in HBSS, and resuspended in growth medium (D-MEM, 15% horse serum, 2.5% fetal calf serum, 2 mM glutamine and penicillin/streptomycin/amphotericin; Sigma). The yield was typically $3\text{--}4 \times 10^6$ cells per AS pituitary, $2\text{--}2.5 \times 10^6$ cells per *dw/dw* pituitary. Cells were cultured in 24-well plates at a density of one quarter pituitary equivalent/1 ml well, except when AS and *dw/dw* cells were combined in different proportions, in which case each mixture was seeded at

5×10^5 cells per well. For immunocytochemistry, cells were cultured in wells containing poly-L-lysine-coated coverslips. The culture medium was changed on day 2 or 3 and cells examined on day 5. In some experiments, the medium was aspirated on day 5, the cells washed twice and incubated with warmed D-MEM with or without GHRH (human GHRH ^{Nleu}27(1–29)NH₂; Ferring AB) for 30–180 min, after which the supernatants and cells were saved for analyses.

FACS

Analysis Anterior pituitaries were enzymically dispersed individually as described above except that trypsin was omitted. Dispersal volumes were 0.5 ml for 9- to 23-day pituitaries and 1.5 ml for 45- to 144-day pituitaries. After washing, the cells were resuspended in FACS buffer (10 g/l NaCl, 0.25 g/l KCl, 1.37 g/l Na₂HPO₄, 0.25 g/l KH₂PO₄, 1 g/l BSA) + 0.1% sodium azide, centrifuged at 300 *g* and fixed in 50 µl 4% paraformaldehyde for 5 min on ice. After washing in FACS buffer+azide, cells were permeabilised by the addition 0.2% saponin (Sigma), centrifuged (130 *g* for 5 min) and resuspended on ice in 50 µl FACS buffer+saponin, containing primary antibodies (monkey anti-rGH (1:2000) and/or rabbit anti-rPRL (1:8000) or a non-immune serum control). After 30 min, the cells were washed and 50 µl secondary antibody diluted in FACS buffer+saponin were added. These were (i) fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG (1:20; Dako) for single PRL staining; (ii) phycoerythrin (PE)-conjugated goat anti-human IgG (1:20; Sigma) for single GH staining; and (iii) FITC-conjugated swine anti-rabbit IgG and biotinylated goat anti-human-IgG (1:200; Vector Laboratories), followed by 50 µl PE-streptavidin (1:1000; Vector Laboratories) for double staining. After 30 min, cells were washed once in FACS buffer+saponin, and twice more in FACS buffer+azide.

Cells were analysed on a FACS Vantage or FACS Star Plus (Becton-Dickinson, Mountain View, CA, USA) and data from $1-2 \times 10^4$ cells collected for each sample. For each age group, negative controls and single-stained controls were run in parallel to correct for crossover between channels and determine quadrant boundaries. Data were analysed using WinMDI software. The population of cells was electronically gated to exclude cell debris and red blood cells, and the data plotted with FITC fluorescence (PRL) on the *x*-axis and PE fluorescence (GH) on the *y*-axis.

Cell sorting Under sterile conditions, cells dispersed from 18 anterior pituitaries were centrifuged at 300 *g* for 5 min, resuspended in 1 ml FACS buffer, layered onto 15 ml 4% BSA in FACS buffer and centrifuged for 5 min at 100 *g* to remove blood cells and debris. Most of the cell

population was incubated on ice for 30 min in monkey anti-rat GH (1:2000) in FACS buffer while an aliquot of 10^6 cells was incubated in non-immune monkey serum (1:2000) as a negative control. After 30 min, the cells were washed in FACS buffer, centrifuged (300 *g* for 5 min) and resuspended in PE-conjugated goat anti-human IgG (1:20). After 30 min, they were washed and resuspended in sterile culture medium (10^6 /ml). Another aliquot of cells was removed (unsorted population), and the remainder sterile sorted to collect GH-enriched and GH-depleted cell populations gated by comparison with the negative control. The three populations of cells were then placed in tissue culture as described above.

Analysis of pituitary extracts

Anterior pituitaries from 10-week-old male and female AS and *dw/dw* rats ($n=5-7$) were homogenised on ice in PBS containing peptidase inhibitors (Complete Peptidase Inhibitor Cocktail; Roche Products), frozen and thawed, centrifuged (5 min at 13 000 *g*), and the supernatant stored at -20°C . Protein contents were determined by a modification of the method of Lowry *et al.* (1951) using BSA as standard. Rat GH and PRL were assayed by RIA using reagents supplied by NIDDK as previously described (Clark *et al.* 1987, Carmignac & Robinson 1990).

For measurement of cAMP, cells were extracted in 500 µl 95% ethanol/0.1 M HCl (Marley *et al.* 1991) at -20°C overnight. They were then transferred to a microfuge tube, together with a 500 µl acid/alcohol rinse. After centrifugation (13 000 *g* for 5 min), 500 µl supernatant were evaporated to dryness under vacuum, and resuspended in PBS. Samples and standards (0.1–800 fmol/100 µl cAMP; Sigma) were acetylated by addition of 2% v/v triethylamine (Sigma) and 1% v/v acetic anhydride (Sigma) and subjected to RIA following the NIDDK protocol for antibody CV-27. Antibody (1:400 000) and tracer (¹²⁵I-labelled 2-O-monosuccinyl cAMP methyl ester; Sigma) were added in a final volume of 250 µl buffer (50 mM sodium acetate + 0.5% BSA, pH 6.2) and incubated overnight at 4°C . Bound and free fractions were separated by adding 500 µl polyethylene glycol, the tubes centrifuged and the radioactivity in the pellets counted.

Statistical analysis

Results are presented as means $\pm$ S.E.M. unless otherwise stated. Statistical analysis was carried out using Instat 2.01. Where two groups were compared, Student's *t*-test or the Alternate Welch *t*-test was used. Where multiple groups were compared, the data were subjected to ANOVA and tested using Bonferroni's comparison of selected pairs. Dose-response curves were post-tested for linear trend and compared using Dunnett's test.

Figure 1 GH and PRL contents and cell numbers in 10 week AS and *dw/dw* rat pituitaries. GH (A) and PRL (B) contents of AS and *dw/dw* rat anterior pituitaries measured by RIA ($n=5-7$). GH (C) and PRL (D) cell numbers determined by immunocytochemistry ($n=3$). Asterisks indicate result of Bonferroni's post-hoc test (a: AS vs *dw/dw*, b: male vs female). * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Results

In vivo experiments

Pituitary GH and PRL contents in adult *dw/dw* and normal animals were compared by RIA (Fig. 1A and B). The profound reduction in GH in *dw/dw* rats was accompanied by an increase in PRL content, significantly so in females. As expected, pituitary PRL content was higher in females than in males for both strains. Western blots of SDS-PAGE gels of 10 µg protein from AS male and *dw/dw* male anterior pituitary extracts confirmed the decrease in GH, and marked increase in PRL (data not shown).

Double immunocytochemistry and cell counting for GH and PRL on anterior pituitary sections from 10-week-old AS and *dw/dw* rats showed a lower proportion of GH⁺ve cells and a higher proportion of PRL⁺ve cells in both male and female *dw/dw* rats compared with AS rats (Fig. 1C and D). In both strains, females had a significantly higher percentage of PRL⁺ve cells than males, whereas the percentage of GH⁺ve cells did not differ significantly between the sexes.

To study PRL and GH cell differences more quantitatively, a two-colour FACS method was developed for

pituitary cells isolated from *dw/dw* and AS rats. Figure 2 shows examples of scatter characteristics from male AS and *dw/dw* pituitaries at different ages. Forward scatter (x-axis) is a measure of cell size, while side scatter (y-axis) relates to the complexity or granularity of the cell. In both strains, side scatter increased with age as the cells became more granular, but no differences in scatter characteristics were observed between AS and *dw/dw* pituitary cell populations.

Figure 3 illustrates typical fluorescence profiles of cells from male AS and *dw/dw* pituitaries at different ages. The upper left quadrant shows 'GH-only' cells (note the marked reduction in somatotrophs in the *dw/dw* compared with AS rats) whilst the lower right quadrant shows 'PRL-only' cells (showing a higher proportion of lactotrophs in the *dw/dw* rats). The upper right quadrant shows cells staining for both GH and PRL (mammosomatotrophs).

Quantitative FACS analysis was performed on pituitaries from groups of *dw/dw* and AS male and female rats from 9 to 144 days. In the younger rats the GH⁺ve, PRL⁺ve and double⁺ve populations were more clearly separated into the quadrants (see Fig. 3), so the data could be obtained directly from two-colour analyses. In 45–144 day rats, where the heterogeneity of the scatter was greater

Figure 2 Examples of scatter characteristics of dispersed pituitary cells from AS and *dw/dw* males at 9 and 70 days of age. Side scatter (SSC) is shown on the y-axis, while forward scatter (FSC) is shown on the x-axis.

Figure 3 Examples of FACS fluorescence plots of stained pituitary cells from AS and *dw/dw* males at 9 and 70 days of age. PE fluorescence (GH) is shown on the y-axis, while FITC fluorescence (PRL) is shown on the x-axis.

(see Fig. 2) and the populations fell less easily into quadrants (see Fig. 3), the 1.5 ml cell dispersion was divided into three aliquots. Single-colour FACS runs were first performed to count the total numbers of GH⁺ or PRL⁺ cells. The double⁺ mammosomatotrophs (i.e. both GH⁺ and PRL⁺ cells) were then counted by performing a separate two-colour FACS run. The GH-only (i.e. somatotroph) and PRL-only (i.e. lactotroph) populations were then calculated by subtracting the number of double⁺ mammosomatotrophs from the total GH⁺ or total PRL⁺ cell counts.

The FACS results are summarised in Fig. 4. They confirmed that the percentage of GH⁺ cells was significantly lower in both male and female *dw/dw* rats at all ages studied, compared with normal AS rats. There were also significantly fewer GH⁺ cells in females than in males at 16 and 45 days. FACS analysis also showed clearly that at all ages, both male and female *dw/dw* rats had a higher percentage of total PRL⁺ cells than their normal AS counterparts. At 9 days the percentage of total PRL⁺

cells was 5-fold higher, and that of PRL-only lactotrophs 8- to 10-fold higher in male and female *dw/dw* rats than in AS rats. This difference became less marked with age, being 3.5-fold at 23 days and <2-fold at 144 days. *dw/dw* males and females had a significantly higher percentage of mammosomatotrophs than AS rats at 9 days, similar percentages at other ages, but a significantly lower percentage than AS rats by 144 days. At 70 days, females of both strains also had a significantly lower percentage of mammosomatotrophs than their respective males, although the mammosomatotroph percentage tended to increase with age in both strains. We can conclude that the increase in PRL⁺ cells in *dw/dw* rats is primarily due to lactotrophs, and not to an increased mammosomatotroph population.

Figure 5 shows the ratio of total GH⁺/PRL⁺ cells (Fig. 5A), and GH-only and PRL-only cells (Fig. 5B) in *dw/dw* and AS rats at different ages, determined by FACS analysis. Allowing for a 40% reduction in total pituitary cell number in *dw/dw* rats, the increased percentage of total PRL⁺ cells represents a real increase in cell number

Figure 4 GH, PRL and double staining cells during development of AS and *dw/dw* rats. Dispersed anterior pituitary cells were stained for GH and PRL and analysed by FACS. Asterisks indicate result of Bonferroni's post-hoc test ($n=3-8$; a: GH-only-AS vs *dw/dw*, b: GH-only-male vs female, c: double⁺-AS vs *dw/dw*, d: double⁺-male vs female, e: PRL-only-AS vs *dw/dw*, f: PRL-only-male vs female). * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Figure 5 Relative GH⁺ and PRL⁺ cell numbers. The percentages of total GH⁺ and PRL⁺ cells (A), and GH-only and PRL-only cells (B), were converted to the ratio of each cell type in *dw/dw*:AS pituitaries allowing for the 40% reduction in pituitary cell number in the *dw/dw*. The horizontal line indicates a ratio of 1, where the cell numbers in *dw/dw* and AS pituitaries would be equal.

before 45 days (Fig. 5A), a difference even more marked when comparing PRL-only lactotrophs, especially in females (Fig. 5B). The number of GH⁺ cells was already reduced by 9 days in *dw/dw* rats, and this somatotroph hypoplasia was maintained throughout the study.

In vitro experiments

Pituitary cells from male AS and *dw/dw* rats were dispersed and maintained in culture for 5 days and then immunostained for GH and PRL. There were markedly fewer GH⁺ cells in cultures from *dw/dw* rats than from AS rats after 5 days in culture (AS: $24.16 \pm 1.64\%$ vs *dw/dw*: $8.39 \pm 1.14\%$; $P < 0.001$), but there was no significant difference between the PRL⁺ cell populations (AS: 37.12 ± 2.56 vs *dw/dw*: 34.04 ± 4.00).

Similar cultures of AS or *dw/dw* cells were exposed to a wide range of GHRH concentrations for 3 h and the GH released into the medium measured by RIA (Fig. 6A and

Figure 6 GHRH dose response in primary pituitary cultures. Primary anterior pituitary cultures from male AS and *dw/dw* rats were incubated for 3 h with 0.001–1000 nM GHRH (or control) and the medium was then assayed for GH. The results are displayed as ng/well (A) and fold increase over basal secretion (B). Asterisks indicate result of Dunnett's multiple comparison (see text for significance levels of *, **, ***) (vs basal; $n=3$ per dose).

B). GHRH stimulated GH release from cells from both strains. The response amplitude and sensitivity to GHRH differed between strains (ED_{50} 0.01–0.03 nM for AS cultures vs 0.3–1 nM for *dw/dw*; Fig. 6A), but this difference was less dramatic after taking into account the lower basal release and hormone contents in the *dw/dw* cells (Fig. 6B). A maximal concentration of 100 nM GHRH induced a 5-fold increase in GH release from AS cells, compared with a 3.5-fold increase from *dw/dw* cells.

Figure 7 Effect of GHRH on cAMP accumulation in primary anterior pituitary cultures from AS and *dw/dw* rats. (A) Primary cultures of male AS and *dw/dw* anterior pituitary cells were treated for 30 min with 0.001–1000 nM GHRH (or control) and the cAMP content measured by RIA. Asterisks indicate result of Dunnett's multiple comparison (see text for significance levels of *, **, ***) (vs basal; $n=3$ per dose). (B) Anterior pituitary cells from male AS and *dw/dw* rats were mixed in various proportions and cultured at 500 000 cells/well for 5 days. The cells were treated with 10 nM GHRH (or control) for 30 min, extracted and assayed for cAMP. Asterisks indicate result of Alternate Welch *t*-test (see text for significance levels of *, **, ***) ($n=3$; control vs GHRH for each cell mixture).

PRL release from *dw/dw* cultures was similar to that of AS cultures (AS: 24.33 ± 2.29 vs *dw/dw*: 20.08 ± 1.41 ng/well at 3 h), and PRL content in both AS and *dw/dw* cultures was unaffected by GHRH exposure (data not shown).

Figure 7A compares cAMP levels in extracts from male AS and *dw/dw* pituitary cell cultures exposed to GHRH for 30 min. GHRH induced a dose-dependent cAMP response in cell cultures from AS rats, although this required higher GHRH concentrations than was necessary to induce GH release (see Fig. 6A). GHRH completely failed to increase cAMP in *dw/dw* rat cultures (Fig. 7A).

To ensure that this did not simply reflect the lower abundance of GH⁺ cells in *dw/dw* cultures, further experiments were performed in which *dw/dw* cells were co-cultured with different percentages of AS cells and their responses to GHRH measured (Fig. 7B). The cAMP response to GHRH increased in proportion to the percentage of AS cells in the mixed cultures, but as little as 5% AS:95% *dw/dw* cell mixture (equivalent to 2% AS somatotrophs), was sufficient to exhibit a significant cAMP response to GHRH (Fig. 7B).

Finally, pituitary cell pools were prepared enriched or depleted in somatotrophs from *dw/dw* pituitaries by FACS sorting prior to culture. Figure 8A shows an experiment in which GH⁺ cell populations were enriched to 45% (i.e. similar to an AS pituitary), or depleted to 1.3% compared with the unsorted starting population (10%; $P<0.001$), and then maintained in culture for 5 days. Note, however, that 60% of 70 day male *dw/dw* GH⁺ cells also express PRL (see Fig. 4) so many of the GH-enriched population are PRL⁺. As expected, both basal and GHRH-stimulated GH release were higher in the GH-enriched population and lower in the GH-depleted populations compared with the unsorted population (Fig. 8B), but even with a *dw/dw* somatotroph population enriched to the same proportions as in an AS culture, GHRH failed to increase cAMP accumulation in *dw/dw* cells (Fig. 8C). This result shows that failure to increase cAMP accumulation by GHRH is an intrinsic deficit in *dw/dw* somatotrophs and is not due to a reduced representation of somatotrophs in such cultures.

Discussion

The *dw/dw* rat strain was initially characterised in 1988 (Charlton *et al.* 1988) and has proved useful as a model of specific GH deficiency (Carmignac *et al.* 1993, 1998, Butler *et al.* 1994, Pellegrini *et al.* 1997, Tei *et al.* 2000, Forwood *et al.* 2001). Although its profound GH deficiency is well established, its PRL status is less clear. Our initial study compared PRL content in a few *dw/dw* and heterozygous normal littermates, and showed no apparent PRL deficiency (Charlton *et al.* 1988). In a later study, pituitary PRL content, measured over a wider age range in male *dw/dw* animals, was similar to, or in a few cases higher than, heterozygous littermates (Bartlett *et al.* 1990). More recently, we noted that the PRL responses to the GH secretagogue GHRP-6 were surprisingly large in *dw/dw* females, were abolished by ovariectomy, and could be induced in males given oestrogen (Carmignac *et al.* 1998), all treatments known to affect lactotroph numbers. We confirmed an increased pituitary PRL storage in male *dw/dw* rats, but found that it was not associated with increased PRL secretion, since basal circulating PRL levels were normal in *dw/dw* rats, and no changes in dopamine (DA) fluorescence were seen in their hypothalamic tuberoinfundibular DA neurons (Thomas *et al.* 1999). We

Figure 8 Functional analysis of FACS sorted male *dw/dw* anterior pituitary cells. (A) Unsorted, GH-enriched and GH-depleted cells were plated on poly-L-lysine-coated coverslips and cultured overnight. Two coverslips of each cell population were immunostained for GH and PRL and counterstained with haematoxylin. GH⁺ cells were counted from four areas of 1 mm² on each of the two coverslips per group. Asterisks indicate result of Bonferroni's post-hoc test (see text for significance levels of *, **, ***): (a) sorted vs unsorted, (b) GH-enriched vs GH-depleted). (B) Unsorted, GH-enriched and GH-depleted cell populations were cultured for 5 days and then treated with 10 nM GHRH (or control) for 3 h in duplicate wells. The medium was assayed for GH and PRL. (C) Unsorted, GH-enriched and GH-depleted cells were cultured as above. After treatment with 10 nM GHRH (or control) for 30 min in duplicate wells, the cells were extracted and assayed for cAMP.

therefore performed the current studies to investigate the cellular basis of the increased PRL reserves in *dw/dw* rats.

Kineman *et al.* (1989) had earlier observed an increased percentage of PRL secreting cells in *dw/dw* rats ($52.4 \pm 4.6\%$ vs $33.7 \pm 3.7\%$ in controls) but attributed this, at least in part, to the paucity of GH cells. In the present study, we counted GH and PRL cells in pituitary sections from both male and female adult *dw/dw* rats. We confirmed an increase in the percentage of total PRL⁺ cells but could not easily quantify the mammosomatotrophs, since these were difficult to identify reliably in *dw/dw* rats, given their much fainter GH staining. To obtain more objective data we developed a two-colour FACS method for assessing PRL and GH cell numbers on dispersed cells. The data we obtained were similar to those from manual cell counts but the FACS method greatly facilitated the examination of much larger numbers of animals and cells and provided more quantitative information on cell populations over a wider range of staining intensities. It also allowed us to quantify and compare the mammosomatotroph populations in both *dw/dw* and normal animals throughout postnatal development.

FACS analysis has previously been used (Hatfield & Hymer 1985, Shinkai *et al.* 1995) to purify and/or count pituitary cells, either after immunostaining or by using cell-specific green fluorescent protein expression in transgenic mice (Magoulas *et al.* 2000). Although more convenient and objective than manual cell counting, it has its own methodological concerns. The staining intensity varies between experiments, so the determination of cut-off limits is done empirically for each set of analyses. We also assume that the inevitable cell losses during dispersal, staining and analysis are similar for GH and PRL cells and between the strains.

FACS analysis confirmed that although *dw/dw* rats had a much lower proportion of GH⁺ cells than AS rats at all ages studied, the overall percentage of GH⁺ cells remained relatively stable despite the marked increases in pituitary size, total cell number and GH content which occur over this period (Hoeffler *et al.* 1985, Zeitler *et al.* 1994). The rise in pituitary GH content with age thus reflects an increase in GH cell number and/or GH content per cell, rather than an increased proportion of GH cells in the pituitary, and occurs normally in *dw/dw* rats since their relative GH deficiency vs AS rats remains remarkably constant from birth to late adulthood (Carmignac *et al.* 1993, Zeitler *et al.* 1994).

The FACS results also confirmed that both male and female *dw/dw* rats had a higher proportion of PRL⁺ cells than did normal rats. This disproportion was greatest in early postnatal life and reduced in magnitude throughout adulthood, but remained significant in *dw/dw* rats even at 144 days. Whilst an increased percentage of PRL⁺ cells in adult animals could simply reflect a reduction in GH cells and hence the total cell number, the increase in the younger *dw/dw* animals can only be explained by a true

increase in lactotroph number. For example, since the total cell number in the 9 day *dw/dw* pituitary is about 60% that of AS rats (Zeitler *et al.* 1994), a 5-fold higher percentage of PRL⁺ cells represents a 3-fold increase in absolute terms.

The proportion of mammosomatotrophs was also higher in *dw/dw* than in AS pituitaries at 9 days, but fell gradually with age, reaching a lower percentage than AS rats in late adulthood. A high proportion of mammosomatotrophs at 9 days is in keeping with the data of Hoeffler *et al.* (1985), and Shinkai *et al.* (1995) also found an increase in mammosomatotrophs with age in normal females. At some ages in our study, the females had fewer mammosomatotrophs than males, but we interpret this cautiously since mammosomatotrophs vary with the oestrous cycle (Kineman *et al.* 1991) and this was not monitored in our study. Overall, our FACS data clearly suggest that the mammosomatotroph population is unaffected in *dw/dw* rats, and that it is an increase in lactotrophs that explains their higher pituitary PRL reserves.

It is noteworthy that, while the percentage of mammosomatotrophs did not differ consistently between AS and *dw/dw* pituitaries, the percentage of GH⁺ cells also expressing PRL was much higher in *dw/dw* rats, especially females of 45 days or older. For example, in 144 day female *dw/dw* pituitaries 76% of the GH⁺ cells also contained PRL, vs 22% in controls. This may explain the exaggerated PRL release in response to GHRP-6 in adult females (Carmignac *et al.* 1998).

When pituitary cells are isolated and maintained in culture, GH production decreases whilst PRL production increases (Frawley & Hoeffler 1988). After 5 days in culture, fewer GH⁺ cells remained from *dw/dw* rats compared with AS rats, showing that the somatotroph deficit persists *in vitro*. GHRH released GH from both AS and *dw/dw* cells in culture, but the latter required higher doses of GHRH, in line with data reported earlier from *in vitro* (Downs & Frohman 1991) and *in vivo* studies (Carmignac & Robinson 1990). However, after taking into account the lower basal GH release and initial GH stores in the *dw/dw* cultures, the differences in GHRH responsiveness were less marked. The *dw/dw* somatotroph expresses a functional GHRH receptor, but at a lower abundance (Carmignac & Robinson 1990, Carmignac *et al.* 1996, Bennett *et al.* 1997, Zeitler *et al.* 1998), which could explain its lower sensitivity to GHRH. Comparable total numbers of lactotrophs were present in 5 day cultures. Basal PRL release was not elevated in *dw/dw* cultures vs AS cultures, nor did GHRH affect PRL release in either strain.

Neither elevated endogenous GHRH expression (Bennett *et al.* 1997, Pellegrini *et al.* 1997), nor chronic *in vivo* treatment with GHRH (Robinson *et al.* 1988) stimulates GH production in *dw/dw* rats. In an important study, Downs & Frohman (1991) showed that GHRH failed to stimulate a rise in cAMP in *dw/dw* cells, and that

this defect could underlie their somatotroph hypoplasia. We confirmed this observation, but were concerned that this could simply reflect a much smaller amplitude cAMP response from the very few remaining GH cells since these are extremely sparsely represented after cultures of *dw/dw* rat somatotrophs. However, when a *dw/dw* culture was seeded with as few as 2% AS somatotrophs, a GHRH-induced cAMP response could readily be detected, whereas no cAMP response could be detected in a culture highly enriched in *dw/dw* cell somatotrophs by FACS sorting. The defect in cAMP response to GHRH is thus clearly intrinsic to the *dw/dw* cell.

It is noteworthy that this clearly separates the GH secretory response to GHRH from the cAMP response. Since in normal somatotrophs, a rise in cAMP and a GH secretory response occur together following GHRH, and some GH release can be elicited *in vitro* by exposure to cAMP analogues (Stachura *et al.* 1986), it is often assumed that the cAMP and GH secretory responses are causally linked. The ability to mount a robust GH secretory response to GHRH without a detectable cAMP response shows that these mechanisms can be independent. Furthermore, the marked difference in sensitivity of GH release (significant at 0.01 nM GHRH) and cAMP accumulation (significant at 10 nM GHRH) also suggests that alternative pathways might be involved in triggering GH release, even in the normal rat pituitary (Canonica *et al.* 1983, Cronin *et al.* 1984, Cuttler *et al.* 1995). We suggest that the cAMP response to GHRH plays little or no role in GH release, but is essential for stimulation of pituitary transcription factor-1 (Pit-1)-dependent GH synthesis and somatotroph proliferation, the processes which are most compromised in the *dw/dw* rat.

How would this explain the increased lactotroph number and PRL stores in the *dw/dw* rat? As discussed above, almost all other genetic rodent models of GH deficiency show reduced pituitary PRL, usually because the mutation affects a gene coding for transcription factors involved in specifying pituitary cell lineages common to GH and PRL, or necessary for PRL production (Nelson *et al.* 1988, Andersen & Rosenfeld 1994, Sornson *et al.* 1996). A possible exception is the *lit/lit* mouse (Eicher & Beamer 1976), which also lacks a cAMP response to GHRH (Jansson *et al.* 1986). There is surprisingly little direct information on pituitary PRL levels in *lit/lit* mice. Lactotroph morphology (Wilson & Wyatt 1986) and cell density (Lin *et al.* 1993) appear normal, although PRL mRNA abundance is reduced (Wojtkiewicz *et al.* 2002). However, the *lit/lit* mouse has an inactivating mutation in the GHRH receptor, no GH secretory response, a normal GH cell population at birth, and a failure in postnatal somatotroph proliferation (Lin *et al.* 1993). This differs in all respects from the *dw/dw* rat, which has a normal GHRH receptor sequence, a clear GH secretory response to GHRH, a defect in somatotrophs manifest as early as embryonic day 18 (Zeitler *et al.* 1994) but a postnatal

increase in GH cells and GH content which runs parallel with that in normal rats (Carmignac & Robinson (1990) and the present study).

Experimentally induced somatotroph hypoplasia also reduces lactotroph numbers and PRL content, suggesting that there is a close lineage dependence of lactotrophs with somatotrophs and/or their common precursor (Behringer *et al.* 1988, Borrelli *et al.* 1988). Furthermore, GH deficiency secondary to GHRH lack is also accompanied by significant PRL deficiency (Li *et al.* 1996). To the best of our knowledge, the *dw/dw* rat remains the only model in which a profound somatotroph hypoplasia is accompanied by a significant increase in PRL cell number and PRL content.

It is generally assumed that the *dw/dw* defect is restricted to the somatotroph (Charlton *et al.* 1988, Zeitler *et al.* 1994). Our re-evaluation of PRL^{+ve} cells during postnatal development shows that the lactotroph lineage is also clearly affected but whether directly or indirectly remains unclear. Since PRL and GH cell numbers vary inversely in the *dw/dw* animals, and this is manifest already in embryonic life, we suggest that the defect primarily affects a common precursor cell, and has a quantitative, not qualitative, impact in reducing the numbers of somatotrophs produced or retained in their differentiated state, resulting in a higher proportion of cells progressing to lactotrophs. Despite our increasing understanding of the origin of pituitary endocrine cell lineages (Theill & Karin 1993, Andersen & Rosenfeld 1994), we still know little about the mechanisms regulating the numbers of differentiated pituitary endocrine cells. Identification of the mutation in *dw/dw* rats could shed new light on the mechanisms by which the relative proportions of GH and PRL cells are initially specified and subsequently maintained throughout adult life.

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