EXPERIMENTAL STUDY

Enhanced anterior pituitary mitotic response to adrenalectomy after multiple glucocorticoid exposures

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Abstract

Objectives: Glucocorticoid withdrawal in man is associated with transient but sometimes prolonged impairment of hypothalamo–pituitary–adrenal axis secretory responsiveness. This has led to continued concern in the clinical arena. The acute anterior pituitary response to glucocorticoids in the rat includes apoptosis-mediated deletion of a cell population. Whilst continued cell turnover following glucocorticoid withdrawal and the potential for differentiation of uncommitted precursor cells and transdifferentiation of other secretory cell types predicts that, given sufficient time, complete anterior pituitary trophic recovery is likely, this hypothesis has not previously been tested.

Design and methods: We have quantified pituitary mitotic and apoptotic rate, as well as corticotroph number and pro-opiomelanocortin transcript prevalence, together with hypothalamic corticotropin-releasing hormone and vasopressin transcript prevalence 5 weeks after three short dexamethasone treatments each separated by a week. Bilateral adrenalectomy was then carried out as a maximal secretory and trophic stimulus, and the response to a fourth dexamethasone treatment assessed 1 week later.

Results: Anterior pituitary mitotic index was significantly higher in rats previously exposed to dexamethasone compared with age-matched controls exposed to dexamethasone for the first time. No differences were found in the subsequent apoptotic response to a fourth dexamethasone treatment or in the levels of paraventricular corticotrophin-releasing hormone or vasopressin transcripts or pituitary pro-opiomelanocortin transcripts.

Conclusions: These data indicate that full recovery of pituitary mitotic activity takes longer than the recovery of secretory parameters, and suggest that, for several weeks after glucocorticoid exposure, the ability of the pituitary to meet fresh demands placed on it may be suboptimal.

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Introduction

In addition to rapid changes in hormone secretion, the anterior pituitary is able to mount mitotic and apoptotic responses to single and recurrent stimuli and their withdrawal (1, 2). A degree of secretory, mitotic and apoptotic plasticity within the pituitary might enhance responses to recurrent stimuli. Persistence of such changes, however, could result in inappropriate hormone hypo- or hypersecretion. As incidental pituitary microadenomas are common (3), yet involvement of classical proto-oncogenes and tumour suppressors in their pathogenesis is very rare (4, 5), mitotic and apoptotic responses to transient stimuli might also predispose the pituitary to adenoma induction or propagation (4).

It has previously been shown that withdrawal of supraphysiological glucocorticoid treatment results in a period of sustained suppression of one or more components of the hypothalamo–pituitary–adrenal (HPA) axis secretory response in both man (6, 7) and rats (8–10). In man, secretory recovery of the HPA axis following short courses of glucocorticoid appears to be complete within 5–10 days of treatment withdrawal (11) and alternate day glucocorticoid therapy is believed to result in relatively mild suppression of the HPA axis during more chronic exposure (12). Prolonged high dose glucocorticoid exposure, however, has been reported to cause more persistent HPA axis suppression (6, 7). Even so, high doses and prolonged treatments do not necessarily correlate with the severity of subsequent HPA suppression, and the outcome of corticosteroid withdrawal remains unpredictable (6, 13). In these studies, apart from noting ‘atrophy’ of the pituitary gland following dexamethasone treatment, the specific mitotic and apoptotic...
consequences of glucocorticoid withdrawal were not addressed. Yet it is these effects, intuitively, that might be expected to give rise to the more long-term secretory consequences of glucocorticoid treatment.

We have previously shown that treatment of male rats with dexamethasone results in a discrete burst of apoptosis in the anterior pituitary. This event, complete after 72 h, is associated with concurrent suppression of mitosis that persists for the duration of dexamethasone treatment. The amplitude of the apoptotic response is significantly increased if rats are adrenalectomized 6–14 days prior to the start of dexamethasone treatment (14). Whether intact or adrenalectomized, subsequent withdrawal of dexamethasone produces a wave of mitotic activity that is responsible, at least in part, for re-establishing a cell population susceptible to glucocorticoid-induced apoptosis on further dexamethasone exposure. A post-glucocorticoid recovery period of 2 weeks, however, is insufficient for complete restoration of the dexamethasone-sensitive cell population irrespective of whether the first dose of dexamethasone is given to intact or to adrenalectomized animals.

To explore the mitotic and apoptotic responses of the anterior pituitary further, we hypothesized first that the absolute cellular deficit induced by glucocorticoid-induced apoptosis might contribute to the delayed secretory recovery of the HPA axis after glucocorticoid treatment. Secondly, that continued mitotic activity after glucocorticoid exposure would gradually restore cell populations and HPA transcriptional and secretory function to normal.

To test this, we have exposed intact male rats to several courses of supraphysiological dexamethasone and, following a prolonged recovery period, examined the extent to which the anterior pituitary trophic responses to adrenalectomy and one further dexamethasone treatment were affected.

**Materials and methods**

**Animals and treatments**

All animal procedures were carried out in accordance with UK Home Office animal welfare regulations. Male Wistar rats weighing between 100 and 120 g were purchased from Bantin and Kingman Universal Ltd (Hull, Humberside, UK) and allowed to acclimatize for 1 week before the start of a treatment regimen comprising multiple, intermittent high doses of glucocorticoids. Groups of intact rats received daily subcutaneous injections of 50 μg/100 g body weight dexamethasone sodium phosphate in saline (David Bull Labs, Warwick, Warwickshire, UK) for 3 days, together with supplementary dexamethasone in the drinking water (0.75 μg/ml Sigma Chemical Co., Poole, Dorset, UK). Injections were administered between 1300 and 1400 h and control animals received an equal volume of saline. After 3 days, glucocorticoid treatment was withdrawn for the next 7 days. This treatment regimen was repeated twice more, following which animals were left untreated for 5 weeks (Fig. 1).

After the 5-week recovery period, groups of rats were surgically adrenalectomized or sham-operated under anaesthesia with an intraperitoneal injection (1 ml/100 g body weight) of a mixture of 2:2:2 tribromoethanol (2% w/v) in 100% ethanol (8% v/v) and 2-methylbutan-2-ol (1.2% v/v) in 0.9% saline. After a further 7 days, groups of animals received a fourth course of dexamethasone treatment as described above. To control for the effects of ageing on pituitary trophic activity, previously untreated but age-matched groups of rats were adrenalectomized before being exposed to dexamethasone for the first time 7 days later.

Groups of rats were killed by stunning and decapitation at intervals beginning at the end of the

**Figure 1** Schematic representation of the experimental protocols followed.
5-week recovery period and finishing 48 h after the start of the last dexamethasone treatment. Adrenalectomized animals were checked post mortem for remnants of adrenal tissue. Animals were killed between 1200 and 1400 h and immediately after removal from their cages in a separate room adjoining that in which they had been housed. The number of animals in each group was between 4 and 12.

**Preparation of tissue sections**

Immediately after decapitation, brains were quickly frozen on dry ice and stored at −70°C. A series of 12 μm thick coronal brain sections was cut through the paraventricular nucleus at −18°C and thaw mounted onto gelatin-coated slides. Slides were stored at −70°C for *in situ* hybridization histochemistry. Pituitary glands were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 48 h, washed in two changes of fresh PBS and embedded in 1% agar before being processed for paraffin wax embedding. A series of 2 μm thick axial sections were cut from each pituitary for histological analysis, for *in situ* hybridization histochemistry and for corticotrophin (ACTH) immunohistochemistry.

**In situ hybridization histochemistry**

*In situ* hybridization histochemistry was performed as previously described (15, 16). Briefly, frozen brain sections were warmed to room temperature and allowed to dry for 10 min before fixing in 4% formaldehyde in PBS for 10 min. Paraffin wax-embedded pituitary sections were dewaxed in two changes of xylene, rehydrated through a graded series of alcohols, rinsed in water and incubated with 7.5 mg/ml proteinase K in 50 mM Tris (pH 7.5) for 1 h at 37°C. All sections were then washed in PBS and incubated in 0.25% (v/v) acetic anhydride and 1.4% (v/v) triethanolamine in 0.9% saline for 10 min at room temperature. Sections were transferred through 70% (1 min), 80% (1 min), 95% (2 min) and 100% (1 min) ethanol, 100% chloroform (5 min), 100% (1 min) and 95% (1 min) ethanol before being air-dried. Hybridization was carried out using 35S-dATP 3′ end-labelled synthetic 48-mer oligonucleotide probes complementary to either corticotrophin-releasing hormone (CRH) (2 × 10⁵ counts/slide), vasopressin (AVP) (5 × 10⁴ counts/slide) or pro-opiomelanocortin (POMC) (10⁵ counts/slide) mRNA. After hybridization, sections were washed for 1 h in four changes of 1× SSC at 55°C and for a further hour in two changes of 1× SSC at room temperature. Slides were briefly dipped in water then air-dried. Dry hybridized sections were opposed to autoradiography film (Hyperfilm MP; Amersham International plc, Amersham, Bucks, UK) and the resulting images (two autoradiographs per animal) were analyzed densitometrically using the programme ‘NIH Image’. Mean optical density of the entire paraventricular nucleus was analysed for CRH and AVP (using pre-determined optical thresholding to obviate observer bias). For the pituitary, both lateral wings were analysed, in their entirety, with the exception of a thin rim around the very edge of each autoradiograph. Results are expressed as % of controls ± S.E. Representative autoradiographs derived from control and adrenalectomized animals are shown in Fig. 2.

**Image analysis for trophic activity**

Apoptotic and mitotic cell counts were performed on 2 μm thick haematoxylin and eosin-stained rat pituitary sections at 1000× magnification with the aid of a dedicated real-time computer system to tag...
and fully the co-ordinates and trophic assignment of manually identified structures within each tissue section (1). The computerized Highly Optimized Microscope Environment (AxioHOME; Zeiss (17)) used in this study, projects a virtual image of the computer screen which appears fractionally above the actual microscope image and allows different markers to be laid down over apoptotic, mitotic or normal cells by hand. The system retains a cumulative score of the numbers of each cell type counted, together with the co-ordinates of each individually marked cell irrespective of subsequent movement of the microscope stage or changes in the power of the objective lens. Extremely accurate quantification of the various cell types is thus possible for each section studied as areas can be circumscribed at low power (eliminating selection bias), and counted at high power without danger of double scoring. The histological markers that were used to identify apoptotic cells were clusters of two or more apoptotic bodies consisting of extremely dense round or oval structures varying in size from approximately 0.7 to 4 µm and surrounded by normal cells. Earlier stages of apoptosis cannot be visualized using haematoxylin and eosin staining and light microscopy. For each animal, three random areas of approximately 47 000 µm² were scored for the presence of mitotic and apoptotic figures. The sensitivity of detection of mitotic and apoptotic figures throughout the study was consistently high and the error in quantifying the number of normal cells surrounding these events was ±2%. Thus the overall error in estimating the prevalence of these trophic events was around 0.001%. All slides were coded and counted by one blinded observer (L A N).

Results are expressed as a percentage of the total cell numbers counted for each animal and data expressed as means±±S.E.

ACTH immunohistochemistry

Pituitary sections were processed for ACTH immunohistochemistry according to a previously published protocol with minor modifications (1). Briefly, dewaxed and rehydrated sections were incubated in phosphate buffer (buffer) containing 20% methanol, 0.2% Triton X-100 and 1.5% hydrogen peroxide for 30 min to deactivate endogenous peroxidases. Sections were washed in three changes of buffer, blocked in 1% normal horse serum and incubated overnight with monoclonal anti-ACTH (residues 1-24; 1/200 diluted in blocking buffer; Novacastra Labs, Newcastle upon Tyne, Tyne & Wear, UK) at 4 °C. Sections were washed in three changes of buffer and incubated for 1 h at room temperature with biotinylated anti-mouse IgG (Vector Labs, Peterborough, Cambs, UK; 1/200 diluted in blocking serum). Following a further three washes in buffer, sections were incubated with Ready-to-Use Vectastain Elite ABC reagent (PK-7100; Vector Labs) for 30 min at room temperature, rinsed in buffer and developed for 6 min in 3-3’ dianinobenzidine substrate made according to the manufacturer’s instructions (SK-4100; Vector Labs). The resulting brown colour reaction was stopped in water and sections were counterstained with haematoxylin.

Immunopositive cell counts were performed at 400× magnification using the computerized AxioHOME system described above. For each animal, four areas of approximately 75 000 µm² each containing an average of 800 cells were scored as positive or negative for the presence of immunoreactive ACTH. Results are expressed as a percentage of the total cell numbers counted for each animal and data expressed as means±±S.E. No corrections were made for any changes in corticotroph cell size that occurs following adrenalectomy.

Statistics

GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used to perform statistical calculations. Differences between groups were evaluated using one-way ANOVA followed by Tukey–Kramer multiple comparison post tests. P < 0.05 was considered statistically significant.

Results

Effect of dexamethasone treatment on body weight

Successive dexamethasone treatments each resulted in a fall in body weight (Fig. 3). There was no difference between treated and untreated groups in the rate of body weight gain in the periods between each successive treatment, but 5 weeks after the completion of the third dexamethasone exposure body weight remained significantly lower than that of vehicle-treated controls (318±5 g; n = 42 compared with 354±6 g; n = 34; P < 0.001).

CRH, AVP and POMC transcriptional activity

Five weeks after the completion of three courses of intermittent dexamethasone administered to intact animals, CRH and AVP transcripts in the hypothalamic paraventricular nucleus and POMC transcripts in the anterior pituitary were not significantly different from those found in control animals injected with vehicle alone (Fig. 4). Seven days after bilateral adrenalectomy there was a significant six- to eightfold increase in paraventricular CRH transcripts (Fig. 4A), a twofold increase in total paraventricular AVP transcripts (Fig. 4B) and a three- to fourfold increase in pituitary POMC transcripts (Fig. 4C) that were not significantly affected by prior dexamethasone exposure. Dexamethasone challenge beginning 7 days after adrenalectomy
also induced similar reductions in all transcripts irrespective of whether or not the rats had received prior dexamethasone exposure.

**ACTH immunohistochemistry**

Under basal conditions and after using a standard formaldehyde fixation procedure, 5.2 ± 0.34% of anterior pituitary cells were immunohistochemically identifiable corticotrophs and there was no significant difference between control, untreated animals and those that had previously been exposed to dexamethasone (Fig. 5). One week after adrenalectomy, the number of identifiable corticotrophs also increased similarly in both groups of rats (8.7 ± 0.37% vs 8.2 ± 0.41%; Fig. 5). Cell counts were not corrected for possible differences in corticotroph cell size that occur following adrenalectomy (1), as this factor could be expected to affect both groups equally.

**Recovery of anterior pituitary trophic responses**

We have previously shown that when the recovery period after glucocorticoid withdrawal is ≤2 weeks, the peak of anterior pituitary apoptotic activity induced by a second or third exposure to dexamethasone is significantly lower than that seen in adrenalectomized age-matched controls exposed to dexamethasone for the first time (2). This decline occurred irrespective of whether the first dose of dexamethasone is given to adrenalectomized or to intact animals.

We have now examined whether a more prolonged recovery period following glucocorticoid exposure allows the population of anterior pituitary cells deleted by dexamethasone to be fully restored. Five weeks after the withdrawal of the last of three doses of dexamethasone given to intact animals, groups of rats were bilaterally adrenalectomized. One week later the prevalence of mitotic figures in the anterior pituitary of this group of rats was 0.187 ± 0.015% (n = 12) compared with 0.114 ± 0.031% (n = 5) seen in age-matched adrenalectomized animals not previously exposed to dexamethasone (P < 0.05; Fig. 6A). The prevalence of mitotic activity in sham-operated, untreated age-matched animals was 0.057 ± 0.009% (n = 17).

As expected, dexamethasone treatment beginning 1 week after adrenalectomy induced a burst of apoptotic activity in the anterior pituitary within the first 48 h of the start of treatment (Fig. 6B). The peak prevalence in adrenalectomized rats previously exposed to dexamethasone was 0.181 ± 0.014% (n = 12) compared with 0.214 ± 0.020% (n = 9) in age-matched adrenalectomized rats receiving dexamethasone for the first time (not significant; Fig. 6). The prevalence of apoptotic activity in sham-operated, untreated age-matched controls was 0.019 ± 0.006% (n = 17). These data indicate that, in this model, the dexamethasone-sensitive population of anterior pituitary cells can be essentially restored given sufficient time after glucocorticoid withdrawal.

**Discussion**

While there is evidence that perinatal exposure to high dose glucocorticoids may permanently re-programme HPA axis function (18–20), the induction of prolonged or permanent changes in HPA axis function by glucocorticoid exposure in adulthood is more open to speculation. Most studies have used normalization of individual secretory components of the HPA axis following exogenous stimulation to signal complete recovery after glucocorticoid withdrawal and have not considered the underlying role of the mitotic and apoptotic elements of the response. Other than the use of alternate day steroid regimens in children (believed to minimize the long-term effects of glucocorticoids on growth (21)) the cumulative effects of multiple, short courses of glucocorticoid treatments have also not been studied.

The proliferative response of the rat anterior pituitary to bilateral adrenalectomy is well described (22) although the origin and fate of cells contributing to

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**Figure 3** The effect of successive dexamethasone treatments on mean body weight (±S.E.M.).
The post-adrenalectomy mitotic surge remain uncertain (2, 14, 23, 24). Dexamethasone induces a reproducible, temporally constrained burst of apoptotic activity in the anterior pituitary within 24 h of exposure (1, 2). As unequivocal histological signs of apoptosis only appear when nuclear and cytoplasmic contents have been degraded, quantification of hormone transcripts or protein markers cannot be used to determine the prior phenotype of this population of cells. Subsequent withdrawal of dexamethasone elicits a further mitotic response, but this is not sufficient to restore the apoptotically sensitive cell population in adrenalectomized animals within the 2-week time frames of our previous studies (2).

In the current study, we have examined the ability of the anterior pituitary to recover its mitotic response to adrenalectomy and apoptotic response to dexamethasone exposure following three, 72-h exposures to high dose dexamethasone, each a week apart. After a 5-week recovery period, adrenalectomy was used as a maximal mitotic stimulus followed by a single dexamethasone challenge as a maximal apoptotic stimulus. We found that the prevalence of mitotic cells in the anterior pituitary 1 week after adrenalectomy was significantly higher in animals that had been previously exposed to multiple dexamethasone treatments compared with age-matched adrenalectomized animals exposed to dexamethasone for the first time. Thus more than 1 month after a period of repeated exposure to trophic stimuli in the form of recurrent dexamethasone exposure and withdrawal, the anterior pituitary continues to modify its component cell populations. There was no detectable effect of prior dexamethasone exposures on either the baseline number of immunohistochemically identifiable corticotrophs at the end of the 5-week recovery period or in the apparent increase in their numbers following bilateral adrenalectomy.

Our previous work has strongly suggested that apoptotic susceptibility is largely confined to anterior pituitary cells that have recently (i.e. within the previous 2 weeks) entered the cell cycle (14). In the present study, despite the small but significant increase in mitotic activity following adrenalectomy at the end of

Figure 4 Prior dexamethasone (dex) exposure has no effect on the prevalence of paraventricular CRH transcripts (A), paraventricular AVP transcripts (B) and pituitary POMC transcripts (C) following adrenalectomy and one further exposure to dexamethasone beginning 7 days after surgery. Mean deviation from controls is shown as % ± S.E.M., n ≥ 5.

Figure 5 The effects of prior dexamethasone exposure on the proportion of immunocytochemically identified corticotroph cells in the pituitary before and after bilateral adrenalectomy (ADX). Means ± S.E.M. are shown, n ≥ 4. ***P < 0.001 compared with control groups.
the 5-week recovery period, we were unable to demonstrate an increase in the dexamethasone-induced apoptosis at the very end of the experiment. It is possible that the increased mitotic activity in the anterior pituitary of previously dexamethasone-exposed animals was too modest to give rise to a detectable difference in the dexamethasone-induced apoptotically sensitive population. In this experimental paradigm it is more likely, however, that the mitotic response is contributing to cell populations (other than dexamethasone-sensitive apoptotically sensitive cells) that are not directly involved in HPA axis recovery or modulation.

At the time of surgical adrenalectomy a persistent difference in mean body weight between animals previously exposed to dexamethasone and those that had received saline alone was evident. It is possible that indirect effects of dexamethasone on growth unrelated to the HPA axis may also have indirectly modified the pituitary mitotic index.

It is extremely difficult to accurately quantify changes in the population size of functional subsets of pituitary cells as a result of changes in mitotic or apoptotic activity. Overall changes amount to a fraction of 1% and the trophic stimuli used markedly affect intracellular peptide levels and hence immunohistochemical sensitivity.

In this comparative quantification study, we have used direct visual identification of apoptotic and mitotic figures based on well-validated morphological criteria to quantify changes in the prevalence of trophic events (1, 2, 25). This method consistently but predictably underestimates the absolute numbers of cells that will be at any earlier stages of either of these processes. Nevertheless, the use of carefully standardized fixation, embedding, cutting and staining methods allows accurate quantification of trophic events and valid comparisons between groups to be made. Direct visual identification with the aid of a computerized tag and tally aid to the manual cell counting used in this study also has the advantage that all types of cells and trophic activity can be identified on the same section and at the same time. The available alternatives for use in tissue sections, such as TUNEL or detection of activated caspases for apoptosis, and detection of incorporated bromodeoxyuridine or Ki-67 for proliferating cells, depend on immunocytochemical methods that necessitate a more subjective and less definitive approach to the identification of positive cells.

There were no significant effects of prior dexamethasone exposure on the levels of paraventricular CRH or AVP transcripts and pituitary POMC transcripts measured at the end of the 5-week recovery period or 1 week after subsequent adrenalectomy. These data support the results of other groups studying the earlier recovery of secretory components of the HPA axis following glucocorticoid administration in rats (8–10).

In summary, the present study has demonstrated that restoration of normal levels of mitotic activity in the anterior pituitary remains incomplete 5 weeks after repeated glucocorticoid exposures. These data indicate that glucocorticoid exposure is associated with subtle changes in pituitary trophic activity that persist after the transcriptional and secretory activity of the HPA axis appears to be restored to normal.

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