Temporally Sensitive Trophic Responsiveness of the Adrenalectomized Rat Anterior Pituitary to Dexamethasone Challenge: Relationship between Mitotic Activity and Apoptotic Sensitivity

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Depending on timing and dose, exogenous glucocorticoids induce a wave of apoptosis in the adult rat anterior pituitary, a response that is enhanced by adrenalectomy. In this study, we show that the size of the glucocorticoid-sensitive apoptotic population progressively increases during the week following surgical adrenalectomy, plateaus for a further week, then spontaneously declines to levels seen in intact animals by 4 wk. Mitotic activity, in contrast, rises rapidly post adrenalectomy but returns to baseline within 2 wk. Increased mitotic activity precedes the increase in the population of cells that undergo glucocorticoid-induced apoptosis and the subsequent decline in mitotic activity precedes the decline in apoptotic sensitivity despite persistent elevation of hypothalamic CRH and pituitary proopiomelanocortin transcripts. If glucocorticoid exposure is delayed until 4 wk post adrenalectomy when the apoptotic response has returned to baseline, glucocorticoid withdrawal, by transiently increasing mitotic activity, again primes the formation of an expanded glucocorticoid-sensitive apoptotic cell population. These data suggest that apoptotic sensitivity is largely confined to cells that have recently entered the cell cycle. This observation is further corroborated by demonstrating an abrupt glucocorticoid-induced step-down in the bromodeoxyuridine-labeling index to basal levels in rats given daily injections of bromodeoxyuridine during the week following adrenalectomy. (Endocrinology 144: 212–219, 2003)

THROUGHOUT THE LIFE of an animal, the anterior pituitary has to respond continuously to transient but often repeated stimuli such as physiological and psychological stresses, pregnancy, and weaning. The repertoire of pituitary responses encompasses not only changes in secretory capacity of existing cells but also changes in mitotic and/or apoptotic (i.e. trophic) activity. When a stimulus is withdrawn, it is assumed that the pituitary essentially restores itself to its prestimulated state. A degree of secretory and trophic plasticity within the pituitary, however, may allow for the delivery of enhanced responses to recurrent stimuli. Equally, persistence of changes may predispose the pituitary to trophic anomalies and account for the high prevalence of microadenomas (1). Previous studies using animal models have shown that the young adult rat anterior pituitary is indeed trophically active even under unstimulated conditions and that its apparent trophic quiescence results almost entirely from the fleeting nature of overt histological evidence of cell death and division and the consequent difficulties in accurately quantifying these trophic events (2, 3).

The cell proliferative response of the rat anterior pituitary following the extreme stimulus of bilateral adrenalectomy without concomitant glucocorticoid replacement is well documented and appears to result in an absolute increase in the number of cells transcribing the proopiomelanocortin (POMC) gene (4–9). Under these circumstances, our own data and the work of others suggest that the origin of the cells entering the cell cycle is a combination of mature corticotrophs and perhaps other hormone-secreting cells together with a number of more immature or pluripotent stem cells (2, 8–10). In this model, the elevation of mitotic activity above basal levels is not maintained despite both the continued absence of glucocorticoid feedback and sustained elevation of CRH transcripts, so that by 2 wk after surgery, mitotic rate has returned to preoperative levels (2, 11). The intact rat pituitary also contains a specific subpopulation of cells, the functional identification of which remains elusive, that undergoes apoptosis in response to the in vivo administration of the synthetic glucocorticoid dexamethasone. We have previously shown that the size of this population increases 3-fold between 1 and 2 wk after bilateral adrenalectomy (2, 12).

To further clarify the temporal relationships between physiological change and pituitary trophic responses, we have in the present study determined the precise timing of the changes in size of the dexamethasone-sensitive apoptotic cell population following adrenalectomy and correlated these changes with fluctuations in the rate of mitosis and changes in pituitary POMC transcript levels and paraventricular CRH transcripts during the first 28 d post adrenalectomy. We have also examined the relationship between mitotic activity and apoptotic sensitivity at the single cell level and addressed the possibility that in chronically adrenalectomized animals, pituitary mitotic activity, and apoptotic responsiveness can be restored by using dexamethasone withdrawal as a surrogate for surgical adrenalectomy.
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, UK) and allowed to acclimatize for 1 week before being surgically adrenalectomized or sham-operated under anesthesia with an ip injection (1ml/100 g body weight) of a mixture of 2:2:2 tribromoethanol (2% wt/vol) in 100% ethanol (8% vol/vol) and 2-methylbutan-2-ol (1.2% vol/vol) in 0.9% saline. Rats were adrenalectomized between 0800 and 1200 h and given 0.9% saline to drink following surgery. Adrenalectomy was performed between 1300 and 1400 h for up to the first 3 days of glucocorticoid treatment.

Control animals received an equivalent volume of saline.

To examine the effects of glucocorticoid withdrawal and repeated exposure to dexamethasone on pituitary trophic activity following long-term adrenalectomy, groups of rats that had been adrenalectomized 28 days previously received dexamethasone for 14 days as described above. Dexamethasone was then withdrawn for 14 days before a second dexamethasone exposure was carried out.

To follow cumulative changes in the number of recently divided anterior pituitary cells, additional groups of rats received daily ip injections of bromodeoxyuridine (BrDU; 10 mg/ml in 0.007 M NaOH/0.9% NaCl). Roche, Welwyn Garden City, Hertfordshire, UK) at a dose of 200 mg/kg body weight. Injections were started on the day of adrenalectomy or sham surgery and continued for 5 days.

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 were cut through the paraventricular nucleus at −18°C and thaw mounted onto gelatin-coated slides. Slides were stored at −70°C before in situ hybridization histochemistry. Pituitary glands were carefully resected and fixed in 4% formaldehyde in PBS for 48 h, washed in two changes of PBS, and embedded in paraffin wax. A series of 2-μm-thick axial sections were cut from each pituitary for histological analysis and for in situ hybridization histochemistry and BrDU immunohistochemistry.

In situ hybridization histochemistry

In situ hybridization histochemistry was performed as previously described (13, 14). Briefly, frozen brain sections were warmed to room temperature and allowed to dry for 10 min before fixing in 4% formamide 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 μg/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% (vol/vol) acetic anhydride and 1.4% (vol/vol) triethanolamine in 0.9% saline for 10 min at room temperature. Sections were then washed in PBS, incubated for 2 h in a mixture of 10% (vol/vol) sodium citrate and 0.01 M citric acid in water, pH 6.0, and incubated for 10 min in a microwave oven on a power setting that maintained the solution just below its boiling point. Sections were then cooled in water to room temperature before permeabilizing for 10 min in 0.01% trypsin (Roche) diluted in 0.1% CaCl2/20 mm Tris buffer (pH 7.5). Following three washes in PBS, the slides were denatured in 2 N HCl in PBS at 60°C for 30 min, washed again in PBS, gently agitated for 20 min in blocking serum (3% normal horse serum, 0.5% Triton X-100 in PBS) and incubated overnight at 4°C with monoclonal anti-BrDU (Sigma; 1:1000 diluted in blocking serum). Sections were washed in three changes of PBS, incubated for 1 h at room temperature with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA; 1:200 diluted in blocking serum), washed again in fresh PBS before blocking endogenous peroxidases for 30 min with 0.6% (vol/vol) hydrogen peroxide in PBS. Following a further three washes in PBS, sections were incubated with a ready-to-use Vectastain Elite ABC reagent (PK-7100; Vector Laboratories) for 30 min at room temperature, rinsed in PBS and developed for 8 min in DAB substrate according to the manufacturer’s instructions (SK-4100; Vector Laboratories). The resulting brown color reaction was stopped in water and sections counterstained with hematoxylin before being mounted in p-xylene-bis(N-pyridinium bromide) (Fig. 2). The number of BrDU-labeled cells was expressed as a percentage of total cell numbers counted (BrDU labeling index: ± SE).

Image analysis for trophic activity

Apoptotic and mitotic cell counts were performed on 2-μm-thick hematoxylin and eosin-stained rat pituitary sections from each pituitary for histological analysis and for in situ hybridization histochemistry. For each animal, three random areas of approximately 47,000 μm2 were scored for the presence of mitotic and apoptotic figures (Fig. 1). The sensitivity of detection of trophic events throughout the study was virtually 100%. The error in quantifying the number of normal cells surrounding these events was 2% or less. Thus, the overall error in estimating the prevalence of trophic events was around 0.001%. All slides were coded and counted by one blinded observer.

Results were expressed as a percentage of the total cell numbers counted for each animal and data expressed as mean ± SE.

BrDU immunohistochemistry

Pituitary sections were processed for BrDU immunohistochemistry according to a previously published protocol with minor modifications (16). Briefly, dewaxed and rehydrated sections were transferred to a hot antigen unmasking solution (0.01 M citric acid in water, pH 6.0) and incubated for 10 min in a microwave oven on a power setting that maintained the solution just below its boiling point. Sections were then cooled in water to room temperature before permeabilizing for 10 min in 0.01% trypsin (Roche) diluted in 0.1% CaCl2/20 mm Tris buffer (pH 7.5). Following three washes in PBS, the slides were denatured in 2 N HCl in PBS at 60°C for 30 min, washed again in PBS, gently agitated for 20 min in blocking serum (3% normal horse serum, 0.5% Triton X-100 in PBS) and incubated overnight at 4°C with monoclonal anti-BrDU (Sigma; 1:1000 diluted in blocking serum). Sections were washed in three changes of PBS, incubated for 1 h at room temperature with biotinylated antismouse IgG (Vector Laboratories, Burlingame, CA; 1:200 diluted in blocking serum), washed again in fresh PBS before blocking endogenous peroxidases for 30 min with 0.6% (vol/vol) hydrogen peroxide in PBS. Following a further three washes in PBS, sections were incubated with a ready-to-use Vectastain Elite ABC reagent (PK-7100; Vector Laboratories) for 30 min at room temperature, rinsed in PBS and developed for 8 min in DAB substrate according to the manufacturer’s instructions (SK-4100; Vector Laboratories). The resulting brown color reaction was stopped in water and sections counterstained with hematoxylin before being mounted in p-xylene-bis(N-pyridinium bromide) (Fig. 2).
Statistics

The statistical software package Prism (GraphPad Software, Inc., San Diego, CA) 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

POMC and CRH transcriptional activity

Bilateral adrenalectomy resulted in a 4-fold increase in CRH transcripts in the hypothalamic paraventricular nucleus by 7 d after surgery (421 ± 37% of control; \( n = 7; P < 0.001 \); Fig. 3A) and a 4-fold increase in pituitary POMC transcripts by 14 d after surgery (376 ± 41% of control; \( n = 6; P < 0.05 \); Fig. 3B). Ten weeks after adrenalectomy, with no additional interventions, both CRH and POMC transcript levels remained significantly higher than their respective controls.

Anterior pituitary trophic responses

The prevalence of apoptotic activity in control, untreated animals was 0.044 ± 0.011%. In the absence of dexamethasone exposure, no significant changes in the prevalence of apoptosis were found at any time point during the month-long study irrespective of whether the rats received anesthetic alone, sham surgery, or bilateral adrenalectomy (data not shown).

As expected, dexamethasone treatment beginning 1 or 2 wk after adrenalectomy induced a discrete, highly significant burst of apoptotic activity in the anterior pituitary within the first 48 h of the start of treatment (2, 12). The effects of a similar duration of dexamethasone exposure commencing at daily intervals during the first week after adrenalectomy and at weekly intervals thereafter for a total of 4 wk on anterior pituitary apoptotic activity (peak prevalence and area under the curve for the first 72 h after the start of dexamethasone treatment) are shown in Fig. 4, A and B.

It can be seen that the amplitude of the apoptotic response to dexamethasone was slightly reduced when treatment began the day after surgery in both adrenalectomized and sham-operated animals. Between 2 and 5 d after surgery, however, the apoptotic response to dexamethasone gradually increased in amplitude in adrenalectomized animals such that by 6 d the prevalence of apoptotic events was significantly higher than that found in intact animals (0.216 ± 0.02%; \( P < 0.01 \)) and comparable with that found at both 1 and 2 wk following adrenalectomy (Fig. 4A). Delaying the start of dexamethasone treatment until either 3 or 4 wk after adrenalectomy resulted in the amplitude of the apoptotic response declining to levels not significantly different from that measured in intact animals.
Sham-operated animals (6.64% vs. 3.79% ± 0.56%; P < 0.01; Fig. 5). Following 48 h of continuous exposure to dexamethasone in animals adrenalectomized 6 d previously, the BrdU labeling index dropped abruptly to 4.28 ± 0.34%, a level not significantly higher than that measured in control animals.

**Restoration of apoptotic sensitivity by induction of a mitotic response**

In striking contrast to peak apoptotic prevalence in animals exposed to dexamethasone beginning 14 d after adrenalectomy (0.276 ± 0.019%; n = 10; P < 0.001), when the first exposure to dexamethasone was delayed until 28 d after adrenalectomy, peak apoptotic prevalence was similar to that seen in intact control animals [0.102 ± 0.018% (n = 6; Fig. 6) compared with 0.082 ± 0.023% (n = 6; NS) respectively]. Irrespective of whether the first exposure to dexamethasone was started 14 d after adrenalectomy (when a large apoptotic response is produced) or 28 d after adrenalectomy (when the apoptotic response is similar to that seen in intact animals), withdrawal of dexamethasone after 2 wk treatment induced a similar, statistically significant mitotic response (P < 0.01 at 5 d after withdrawal, Fig. 6). Furthermore, 2 wk after this point, reexposure to dexamethasone produced a similar apoptotic response in both (0.157 ± 0.025%; n = 6; Fig. 6, compared with 0.147 ± 0.025; n = 6). The amplitude of this apoptotic response was smaller than the first response in the group initially treated 2 wk after adrenalectomy, but larger than the first response in the group in which treatment was delayed until 28 d—an observation that again emphasizes the correlation between the amplitude and timing of mitotic activity and subsequent apoptotic responsiveness.

**Discussion**

Bilateral adrenalectomy in the rat is followed by a prolonged rise in hypothalamic CRH and arginine vasopressin (AVP) transcription and persistent elevation of pituitary POMC transcription with increased translation and secretion of their respective proteins. These changes are associated with limited hypertrophy and hyperplasia of pituitary corticotrophs (reviewed in Ref. 11). Data presented in the present study show that despite direct and indirect consequences of the continued absence of glucocorticoid feedback, by 4 wk after adrenalectomy trophic responses in the anterior pituitary gland have essentially returned to those seen in intact animals in terms of both the prevalence of mitotic figures and the amplitude of the apoptotic response induced by dexamethasone treatment. There therefore appears to be a dissociation between hypothalamic and direct feedback stimulation of pituitary hormone gene expression after adrenalectomy that is relatively sustained, and pituitary trophic activity that, in contrast, is comparatively transient. This dichotomy of responses is not unexpected as a physiological response to prolonged stress seems unlikely to require a persistent proliferative response once sufficient cellular resources have been marshaled to maintain an appropriately increased level of ACTH. In these circumstances a limited trophic but persistent synthetic and secretory response to a change in CRH and AVP levels seems well suited to physiological demands. The question is how these different responses appear to be mediated by the same stimuli. Addi-
tional cytokines, growth or differentiation factors may be called upon to control the balance of proliferation vs. secretory activity. Leukemia inhibitory factor, for example, has been shown in the AtT20 corticotroph cell line to induce a block in cell cycle progression from G1 to S phase while acting synergistically with CRH on POMC transcription (17).

Following adrenalectomy, there is a progressive reduction in pituitary corticotroph CRH-binding capacity and CRH-stimulated cAMP production, which is sustained for at least 9 wk after surgery (18–20). The majority of studies have demonstrated that the level of CRH receptor transcripts is only transiently reduced, however, and although augmented by the absence of glucocorticoids in adrenalectomized rats, returns to control levels between 4 and 14 d after surgery (21–23). These studies suggest that CRH receptor downregulation is related to the high rate of receptor occupancy and internalization in the presence of high levels of hypothalamic CRH and AVP rather than decreased receptor synthesis. The molecular mechanism responsible for the recovery of CRH receptor transcripts to basal levels remains unknown but may be a reflection of the continued absence of glucocorticoids (22) or exposure to altered ratios of CRH.

**Fig. 4.** Dependence of anterior pituitary apoptotic response to dexamethasone on time after adrenalectomy. A, Peak amplitude of apoptotic response to dexamethasone, showing the progressive increase in size of the dexamethasone-sensitive apoptotic cell population during the first 7 d post adrenalectomy, and the subsequent spontaneous decrease to levels seen in intact animals by 28 d. % prevalence mean ± SE. **, P < 0.01; ***, P < 0.001 compared with control (intact animals). B, Peak amplitude of apoptotic response to dexamethasone shown as area under the curve of apoptotic response during the first 72 h of dexamethasone exposure. The inset shows the typical pattern of change in prevalence of apoptotic cells at each time point. C, Correlation of changes in mitotic response following adrenalectomy with peak apoptotic response to dexamethasone, showing the lag between increased mitotic activity and augmentation of the dexamethasone-sensitive cell population and subsequent spontaneous reduction in mitotic activity (with no other treatment at any time), with diminishment of the dexamethasone-sensitive apoptotic cell population. Mean prevalence % ± SE.
animals (see d 28 ptotic response to dexamethasone has declined to that seen in intact dexamethasone withdrawal. By 28 d after adrenalectomy, the apo-
sponse to dexamethasone by induction of a mitotic response through
dexamethasone treatment (Adx
following adrenalectomy. It can be seen that adrenalectomy alone
activity and sensitivity to glucocorticoid-induced apoptosis. Daily
Means
apoptotic cell population contains a number of recently divided cells.
that seen in sham-operated controls (Controls), indicating that the
mitotic cells, temporally constrained as the response declines
3-month-old rats is approximately 150,000 cells. Within a
week of bilateral adrenalectomy, the size of this population
3-month-old rats is approximately 150,000 cells. Within a
week of bilateral adrenalectomy, the size of this population
necessary to induce an apoptotic response (28, 29). Colocal-
and AVP with time (23). The timing of the recovery of CRH
receptor transcripts to basal levels appears to just precede the
reduction in proliferative activity in the anterior pituitary
following adrenalectomy.
The anterior pituitary contains a population of cells that
undergo apoptosis in response to in vivo administration of
dexamethasone (2). The size of this population in intact
3-month-old rats is approximately 150,000 cells. Within a
week of bilateral adrenalectomy, the size of this population
has increased more than 3-fold and remains at this level for
at least a further week (12). In this study, we have shown first
that the adrenalectomy-induced increase in dexamethasone-
sensitive cells is, like the increase in adrenalectomy-induced
mitotic cells, temporally constrained as the response declines
to the level seen in intact animals if first exposure to dexa-
methasone is delayed until 4 wk after surgery. Secondly, we
have shown that the increase in the number of dexametha-
sone-sensitive cells gradually accumulates over the first
week following surgery, paralleling, after a short delay, the
increase in mitotic cells which peaks 6 d after surgery. Sub-
sequently, the decline in numbers of dexamethasone-sensi-
tive cells lags behind the fall in mitotic cells by approximately
1 wk, suggesting that apoptosis only occurs in cells that have
to enter the cell cycle within the previous 2 wk. The close
association between stimulation of mitotic activity and gen-
eration of apoptotically vulnerable cells is also suggested by
the increase in size of the apoptotically sensitive cell popu-
lation following the mitotic response induced by dexameth-
asone withdrawal when first dexamethasone treatment is
delayed until 1 month after adrenalectomy.
To further confirm the association between mitosis and
apoptotic sensitivity at the single cell level, cells entering
S-phase during the week following adrenalectomy were la-
abeled by daily injection of BrdU. Comparison of the anterior
pituitary BrdU labeling indices before and after 2 d of con-
inuous exposure to glucocorticoid demonstrated the ex-
pected abrupt step-down in the labeling index to levels in-
distinguishable from control levels, indicating that apoptosis
occurs largely in cells that had undergone recent mitosis.
BrdU administered at the dose used in this study is not
thought to be inherently toxic and potential dilution of the
label was avoided by the use of daily injections (24). In
addition, the mitotic and apoptotic indices measured in ad-
joining hematoxylin and eosin-stained pituitary sections from
the animals used in these experiments were identical to those
taken from animals not given BrdU. Our data do not yet
allow us to directly determine either the number of newly
formed cells that die following adrenalectomy before dexa-
methasone administration or due to basal cell turnover, or
the precise number of cells that actively contribute to the
dividing cell population.
Technical difficulties have so far made it impossible to
identify the specific nature of apoptotic cells in pituitary
tissue sections using quantification of transcript and/or
protein markers, as unequivocal phenotypic signs of ap-
oposis only appear when nuclear and cytoplasmic contents
have been degraded. The susceptibility of a specific
subpopulation of cells to dexamethasone-induced apopto-
sis is likely to be determined not only by its metabolic state,
but by its specific receptor complement and activated cell
signaling pathways.
Glucocorticoid-mediated apoptosis has been extensively
studied in lymphocytes in which glucocorticoids are able to
induce both G1 cell cycle arrest and apoptosis, although the
molecular mechanisms remain poorly understood and no
single glucocorticoid-regulated genes have yet been impli-
cated (25, 26). It has been suggested that dexamethasone
susceptibility of human T cells to dexamethasone-induced
apoptosis is also cell cycle dependent (27).
It is possible that a change in the number of intracellular
type II glucocorticoid receptors (GR), which in the presence
of ligand result in altered patterns of gene expression and/or
repression in a cell-specific context, is one of the factors
necessary to induce an apoptotic response (28, 29). Colocal-

ization of GR protein and transcripts with individual pituitary hormones has demonstrated that GR is expressed in virtually all ACTH, GH, and folliculostellate cells together with two-thirds of TSH cells and a smaller minority of FSH, LH, and PRL cells (30–32). No overall changes in anterior pituitary GR transcripts following adrenalectomy have been reported (33), but dexamethasone exposure in adrenalec- mized animals resulted in an overall increase in GR transcripts. Positive autoregulation of GR expression, although not common, has also been shown to be a requirement for glucocorticoid-induced cell death in sensitive T cells (29, 34). GR status was not examined in the current study as it would be impossible to specifically quantify any changes within the tiny fraction of cells that undergo apoptosis in response to dexamethasone, not least because these cells cannot be identified before disclosing their dexamethasone-susceptibility by dying.

Thymocytes from adult GR-knockout mice that express a truncated, nonfunctional dexamethasone-binding fragment of the GR are resistant to dexamethasone-induced apoptosis, data that suggest that functional GR is necessary for this particular apoptotic pathway (35). Conversely, increased apoptotic sensitivity of primary thymocytes was observed in response to dexamethasone in mice with an increased GR gene dosage together with significantly reduced levels of CRH, POMC, and basal corticosterone (36).

We have previously shown that at least partial restoration of a trophically sensitive population of cells occurs after dexamethasone withdrawal, as successive exposures to dexamethasone induce successive bursts of apoptosis (12). The amplitudes of these subsequent apoptotic bursts are reduced suggesting either that a population of dexamethasone-sensitive cells is irreversibly deleted or that the pituitary requires a longer time period in which to fully restore its complement of cells. If the first exposure to dexamethasone is delayed until 4 wk after adrenalectomy, the amplitude of the apoptotic response is similar to that seen in intact animals despite the fact that there is no change in the number of immunocytochemically identified corticotrophs present between 7 and 28 d after adrenalectomy (12). However, withdrawal of dexamethasone is able to prime the formation of a larger trophically sensitive population of cells, which is identical in nature to that seen following reexposure in rats treated with dexamethasone for the first time either 1 or 2 wk after surgery. These data also suggest that an apoptotic response is somehow limited to cells that have recently entered the cell cycle, and that the timing of a stimulus or repeated stimuli such as glucocorticoid treatment(s) may be as important as the dose or route of administration in governing the long term consequences for pituitary functionality.

Acknowledgments
We would like to thank the Wellcome Trust for supporting this study and Dr. Heather Cameron for her help in establishing the BrdU protocols.

Received February 28, 2002. Accepted October 8, 2002.

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This work was supported by The Wellcome Trust.

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