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Adrenal Steroids Regulate Postnatal Development of the Rat Dentate Gyrus: I. Effects of Glucocorticoids on Cell Death

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ABSTRACT

The rat dentate gyrus undergoes a period of naturally occurring cell death during the first postnatal week. In the adult rat, removal of circulating adrenal steroids by adrenalectomy is followed by massive death in the granule cell layer, thus raising the possibility that developmental cell death results from low levels of these hormones. Interestingly, the first two postnatal weeks of life in the rat, termed the stress hyporesponsive period, are characterized by very low levels of adrenal steroids. In order to determine whether low levels of adrenal steroids enable developmental cell death to occur in the dentate gyrus, we examined the density of pyknotic and healthy cells in the dentate gyrus of rat pups which received one of the following treatments: (1) injections of the endogenous rat glucocorticoid corticosterone during the first postnatal week, or (2) adrenalectomy at the time when glucocorticoid levels normally rise. Quantitative analysis of the density of pyknotic cells in the granule cell layers revealed significant decreases with corticosterone treatment by the end of the first postnatal week. In these same brains, treatment with corticosterone resulted in a substantial increase in the density of pyknotic cells in the hilus. Adrenalectomy resulted in a significant increase in the density of pyknotic cells in the granule cell layer as well as in the hilus. Despite the dramatic alterations in the density of pyknotic cells with both increases and decreases in glucocorticoid levels, the density of healthy cells remained the same. These observations suggest that glucocorticoids regulate several processes, possibly including neurogenesis and migration, in addition to cell death.

Key words: corticosterone, adrenalectomy, granule cell, hilus, pyknotic cell

During the first postnatal week, the granule cell population of the rat dentate gyrus undergoes a period of substantial cell death, characterized by an increase in the density of pyknotic cells and a decrease in the density of healthy cells (Gould et al., '91). This regressive process seems to play an important role in determining the number of granule cells in the adult. Recent studies performed in the adult rat have demonstrated that granule cells require glucocorticoids for their survival, i.e., removal of circulating glucocorticoids by adrenalectomy results in the degeneration of granule cells in as short a time as three days (Gould et al., '90) and, in some cases, complete obliteration of the granule cell layer by 3–4 months following adrenalectomy (Sloviter et al., '89). Adrenalectomy-induced granule cell degeneration can be prevented by replacing the endogenous rat glucocorticoid corticosterone (Sloviter et al., '89; Gould et al., '90). These observations present the possibility that naturally occurring cell death in the developing dentate gyrus is also regulated by glucocorticoids. Interestingly, the first two postnatal weeks of life in the rat, termed the stress hyporesponsive period, are characterized by very low levels of circulating glucocorticoids (for review, see Sapolsky and Meaney, '86). Since experimentally induced decreases in glucocorticoid levels result in granule cell death in the adult, it is possible that the low levels of glucocorticoids which occur naturally during the first two postnatal weeks enable cell death to proceed during this developmental period.

In order to determine whether low levels of glucocorticoids are necessary for developmental cell death to occur, we examined the density of both pyknotic cells and healthy cells in the dentate gyrus of rat pups subjected to one of the following treatments: (1) injection of corticosterone during the stress hyporesponsive period in order to eliminate the transient decrease in glucocorticoid levels which normally occurs, or (2) adrenalectomy toward the end of the stress hyporesponsive period in order to prevent the natural rise

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in glucocorticoid levels which occurs at this time, i.e., to extend the period when glucocorticoid levels are low.

MATERIALS AND METHODS

Animal treatment and histology

Timed pregnant Sprague-Dawley (Charles River) rats were housed individually in plastic cages and given unlimited access to food and water. At postnatal day 2 (the day after birth), the rat pups were removed from their litters, weighed, and 5 females and 5 males distributed to each dam. From P2 until the time of perfusion, the pups of 8 litters received daily subcutaneous injections of either 5 mg/kg corticosterone in sesame oil or the vehicle alone. This dose of corticosterone was used because it produces blood levels in pups which are in the normal basal adult range one hour following injection (unpublished observation). Both males and females of litters were treated similarly but only the males were used for this study as a previous report has shown no sex differences in developmental cell death in the rat dentate gyrus (Gould et al., '91). Half of the male pups were perfused on P4, the other half on P6. These days were selected because this time frame represents the period when dentate gyrus cell death is maximal (see Gould et al., '91). On the day of perfusion, the pups were weighed, deeply anesthetized with Metofane and transcardially perfused with 10–20 ml 4.0% paraformaldehyde in 0.1 M phosphate buffer and 1.5% (v/v) picric acid (pH 7.4). The brains were dissected from the cranial cavities and placed in a solution having the same composition as the perfusate. The thymus and adrenal glands were removed and weighed following perfusion.

Rat pups from four litters were subjected to either adrenalectomy or sham operation at P15 under Metofane anesthesia. This day was selected because it represents the time when glucocorticoid levels begin to rise in the normal developing rat (Sapolsky and Meaney, '86). Following surgery, the rats were returned to the dam and 0.9% NaCl was provided to the adrenalectomized rats in water bottles with the spouts positioned for easy access by the pups. Seven days following surgery, these rats were anesthetized and perfused as described above. The thymus glands were removed and weighed following perfusion. For the analysis of brains from adrenalectomized rats, only those animals with blood corticosterone levels below 0.4 μg/100 ml, as determined by radioimmunoassay using B21-42 antiserum (Endocrine Science, Tarzana, CA), were selected for analysis.

After postfixation of the brains overnight they were placed into 30% sucrose in PBS for several days. After the brains sank, they were frozen on dry ice and coronal sections 16 μm thick throughout the entire dentate gyrus were cut on a cryostat and thaw-mounted onto gelatinized slides. The sections were then stained for Nissl with a cresyl violet stain and coverslipped under Permount.

Data analysis

The slides containing brain sections for quantitative analysis were coded and the code was not broken until the analysis was finished. For each brain, four sides of selected sections from each of the following three levels throughout the dentate gyrus were selected for analysis: (1) the rostral dentate gyrus where the suprapyramidal blade is not connected to the infrapyramidal blade, (2) the middle dentate gyrus where the suprapyramidal blade and the infrapyramidal blade are joined at the crest region and the dentate gyrus is oriented horizontally beneath the corpus callosum, and (3) the temporal dentate gyrus where the suprapyramidal and infrapyramidal blades are joined at the crest region and the dentate gyrus is oriented obliquely beneath the corpus callosum (for examples of these levels see Gould et al., '91). In order to avoid the necessity of correcting for twice counted cells, the selected sections were always separated by at least 16 μm. For each selected side of each selected section, the numbers of pyknotic or degenerating cells were counted in the suprapyramidal blade, the infrapyramidal blade, and the hilus (1000× magnification). Pyknotic cells were identified by the presence of darkly stained condensed chromatin in granule form, lack of a nuclear membrane and pale or absent cytoplasm (Sengelaub and Finlay, '82). Since the boundaries of the infrapyramidal blade were difficult to discern at P4, the number of pyknotic cells was counted for the infrapyramidal blade and hilus combined at this time. For the suprapyramidal and infrapyramidal blades, the number of healthy cells was also counted. Healthy cells were counted if they possessed distinct boundaries; cell fragments were excluded from the analysis. Since the hilus contains neurons other than granule cells, i.e., mossy cells and CA4 pyramidal cells, the number of healthy cells was not determined for this region.

The cross-sectional areas of the suprapyramidal blade, infrapyramidal blade, and hilus were then determined by tracing the structure on sections from which cell counts were made by use of a camera lucida drawing tube (50× magnification) and the SMI (Southern Micro Instruments) image analysis morphometry program. For each region on each section, the numbers of both pyknotic and healthy cells were then expressed per 10^6 μm^2. A minimum of eight brains were analyzed for each time. In order to determine whether the size of granule cells was affected by developmental manipulations in glucocorticoid levels, the cross sectional cell body area of granule cells in the dentate gyrus was determined by use of a camera lucida drawing tube and the ZIDAS (Zeiss Interactive Digitizing Analysis System, 1000×). For each brain used in the cell count analysis, 40 non-pyknotic granule cells were measured from each of the regions of the dentate gyrus from which pyknotic cell and healthy cell counts were made. A total of 240 cells were measured from each brain in the P6 corticosterone experiment and the adrenalectomy experiment. Since the infrapyramidal blade is not distinguishable at P4, cell body area measurements were only obtained from the suprapyramidal blade (a total of 120 cells were measured per brain at this time point). Means of these variables were determined for each animal for a given region of the dentate gyrus and these data were subjected to unpaired Student's t tests.

RESULTS

Effects of corticosterone treatment and adrenalectomy on rat pups: general observations

Treatment of rat pups with corticosterone resulted in significant decreases in body weight, thymus to body weight ratio and adrenal gland to body weight ratio compared to controls by P6. Adrenalectomy resulted in significant decreases in body weight as well as increases in thymus to body weight ratio compared to sham-operated animals.

TABLE 1. Effects of Corticosterone on the Density of Pyknotic Cells in the P4 Dentate Gyrus[1]

Region	Sham (no. pyknotic cells /10^6 μm^2)	Corticosterone (no. pyknotic cells /10^6 μm^2)
Rostral:		
Suprapyramidal blade	25.9 ± 2.0	30.3 ± 7.0
Infrapyramidal blade/hilus	52.2 ± 7.5	55.1 ± 4.7
Middle:		
Suprapyramidal blade	18.7 ± 4.1	20.4 ± 5.3
Infrapyramidal blade/hilus	25.5 ± 5.7	36.5 ± 6.9
Temporal:		
Suprapyramidal blade	8.2 ± 2.7	9.0 ± 3.0
Infrapyramidal blade/hilus	28.5 ± 5.1	23.3 ± 5.5

[1]Values represent mean ± S.E.M. These data were subjected to two tailed Student's t tests. No significant differences were detected.

TABLE 2. Effects of Corticosterone on the Density of Healthy Cells in the P4 Suprapyramidal Blade[1]

Region	Sham (no. healthy cells/10^6 μm^2)	Corticosterone (no. healthy cells/10^6 μm^2)
Rostral	10496.7 ± 792.3	10003.2 ± 692.5
Middle	10068.1 ± 876.9	9602.8 ± 484.7
Temporal	10311.7 ± 552.9	10427.3 ± 523.9

[1]Values represent mean ± S.E.M. These data were subjected to two tailed Student's t tests. No significant differences were detected.

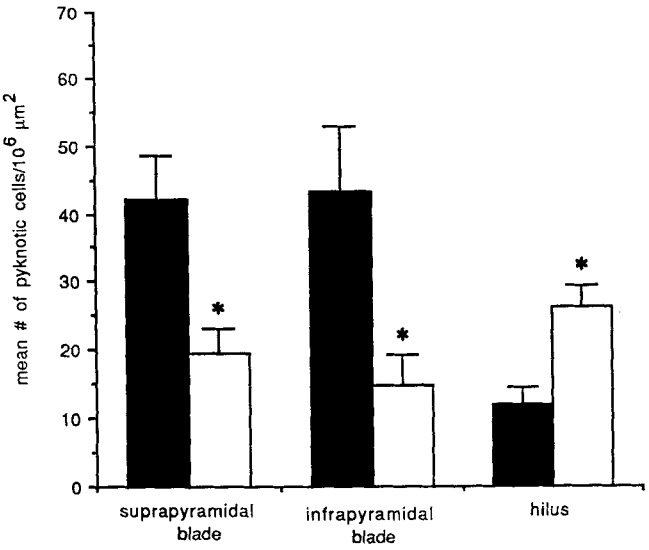

Fig. 1. The density of pyknotic cells in the middle dentate gyrus of sham-injected (solid bar) and corticosterone-injected (open bar) P6 rat pups. Bars represent mean + SEM each obtained from at least four brains. Asterisks represent significant difference from sham ($p < 0.05$).

Effects of corticosterone treatment and adrenalectomy on the developing dentate gyrus

Corticosterone administered daily between P2 and P4 did not affect the density of pyknotic or healthy cells in any level of the dentate gyrus when brains were examined on P4 (Tables 1, 2). However, by P6, elevated levels of circulating corticosterone produced significant decreases in the density of pyknotic cells compared to controls in both the suprapyramidal and infrapyramidal blades at the middle and temporal levels of the dentate gyrus (Figs. 1–3). No significant changes in the density of pyknotic cells were observed at the rostral level in the suprapyramidal blade, infrapyramidal blade or hilus (Fig. 4). In contrast, corticosterone treatment resulted in a significant increase in the density of pyknotic cells in the hilus at the middle level of the dentate gyrus by P6 (Figs. 1, 3, 5). No significant change in the density of pyknotic cells was observed in the temporal hilus with corticosterone treatment (Fig. 2).

Despite the significant changes in the density of pyknotic cells in the granule cell layers and the hilus of the P6 rat with corticosterone treatment, no significant changes in the density of healthy cells were observed at any level throughout the dentate gyrus (Table 3). In addition, corticosterone treatment did not change the cross-sectional area of the suprapyramidal blade, infrapyramidal blade or hilus by P4 or P6. Moreover, no significant differences in the cross-sectional cell body area of granule cells were observed with corticosterone treatment by P4 or P6.

Prevention of the rise in glucocorticoids toward the end of the stress hyporesponsive period, by adrenalectomy at P15, resulted in significant increases in the density of pyknotic cells in the suprapyramidal blade and infrapyramidal blade at rostral, middle, and temporal levels compared to sham-operated rats (Figs. 6–8). In addition, significant increases in the density of pyknotic cells were observed at the rostral and middle levels of the hilus of adrenalectomized rats. Despite this increase in the density of degenerating cells, all regions examined in the dentate gyrus showed no significant changes in the density of healthy cells (Table 4). No changes in the cross-sectional area of the suprapyramidal blade, infrapyramidal blade, or hilus were observed with adrenalectomy at P15. Moreover, no significant differences in the cross-sectional cell body area of granule cells were observed with adrenalectomy.

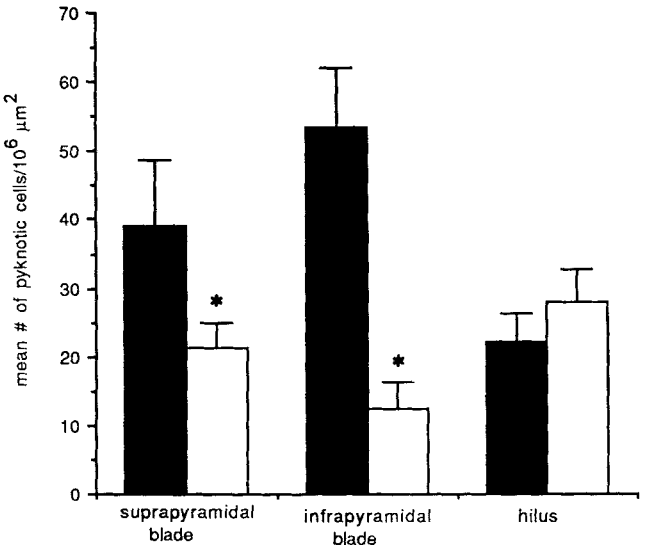

Fig. 2. The density of pyknotic cells in the temporal dentate gyrus of sham-injected (solid bar) and corticosterone-injected (open bar) P6 rat pups. Bars represent mean + SEM each obtained from at least four brains. Asterisks represent significant difference from sham ($p < 0.05$).

DISCUSSION

The results of this study suggest that the low level of glucocorticoids which occurs during the stress hyporespon-

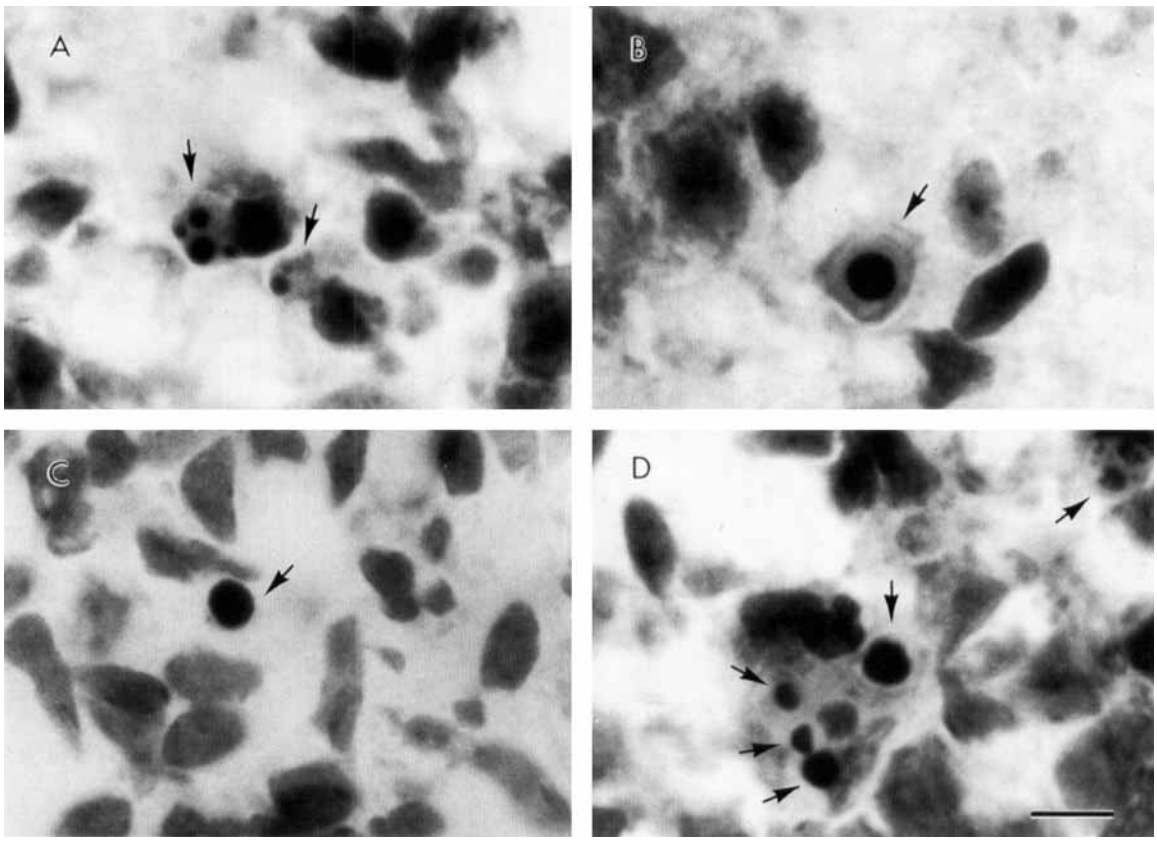

Fig. 3. Representative pyknotic cells in the suprapyramidal blade (**A, C**) and hilus (**B, D**) of sham-injected (A, B) and corticosterone-injected (C, D) P6 rats. Observe the decrease in density of pyknotic cells (arrows) with corticosterone treatment in the suprapyramidal blade and the increase in density of pyknotic cells (arrows) with corticosterone treatment in the hilus. Scale bar in D = 10 μm and applies to all frames.

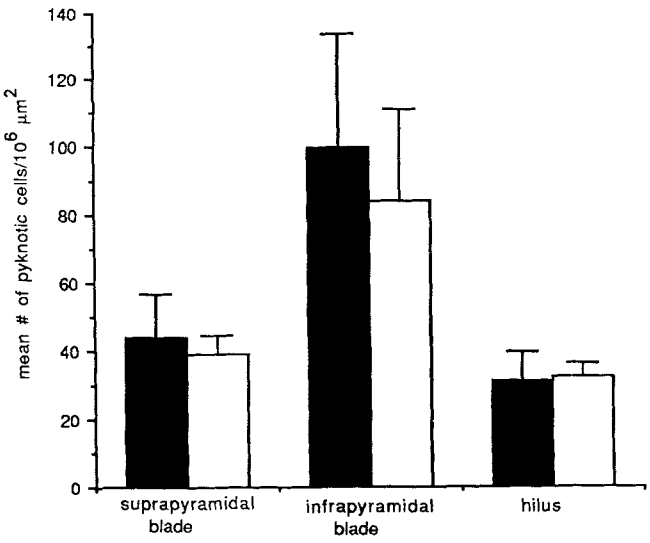

Fig. 4. The density of pyknotic cells in the rostral dentate gyrus of sham-injected (solid bar) and corticosterone-injected (open bar) P6 rat pups. Bars represent mean + SEM each obtained from at least four brains. No significant differences were detected.

sive period is an important factor in regulation of cell death in the developing dentate gyrus. Increased corticosterone levels during the period of maximal cell death, when adrenal steroid levels are normally low, decreased the density of pyknotic cells within the granule cell layer and increased the density of pyknotic cells within the hilus. On the other hand, reduction in the level of circulating corticosterone toward the end of the period of cell death, when adrenal steroid levels are normally rising, increased the density of pyknotic cells throughout the dentate gyrus. Since glucocorticoid manipulations did not significantly affect either cross-sectional cell body area or the cross-sectional area of the granule cell layers or the hilus, it is likely that the changes in density of pyknotic cells observed represent alterations in the absolute numbers of these profiles. Surprisingly, despite the substantial changes in the density of pyknotic cells observed in this study with developmental glucocorticoid manipulations, no significant changes in the density of healthy cells were observed.

Although it is not possible to determine whether the pyknotic cells we observed are neurons or glia, it is likely that those located in the granule cell layer are primarily neurons, as very few glia exist in this portion of the dentate gyrus (Kosaka and Hama, '86).

Possible processes influenced by glucocorticoids during dentate gyrus development

There are at least three not mutually exclusive explanations for the profiles of degenerating and healthy cells we observed in the dentate gyrus following developmental glucocorticoid manipulations. First, glucocorticoids could

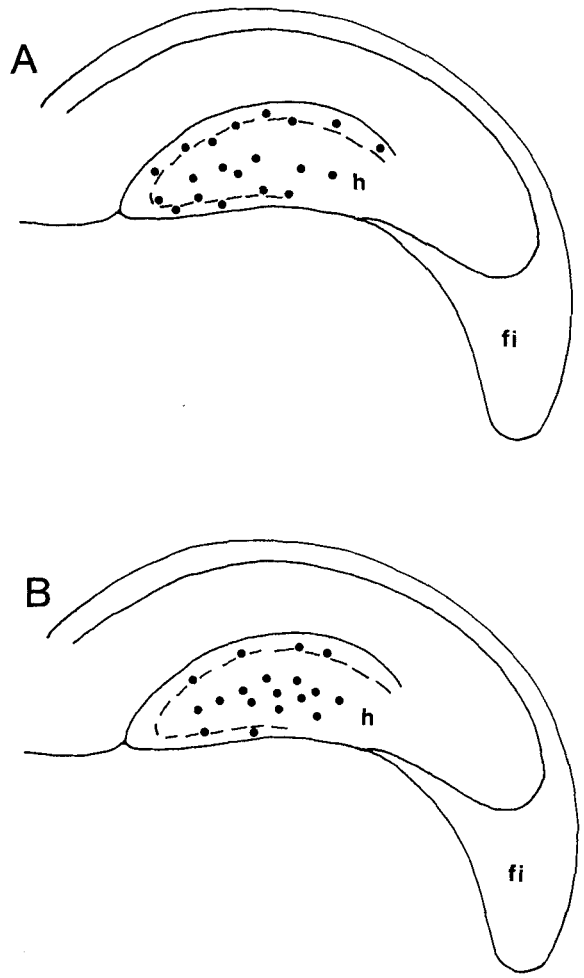

Fig. 5. Templates mapping the distribution of pyknotic cells in the P6 middle dentate gyrus of sham-injected (**A**) and corticosterone-injected (**B**) rats. One dot represents one pyknotic cell. Observe the decrease in pyknotic cell density in the granule cell layers and increase in pyknotic cell density in the hilus with corticosterone treatment. h, hilus, fi, fimbria.

TABLE 3. Effects of Corticosterone Treatment on the Density of Healthy Cells in the P6 Dentate Gyrus[1]

Region	Sham (no. healthy cells/10^6 μm^2)	Corticosterone (no. healthy cells/10^6 μm^2)
Rostral:		
Suprapyramidal blade	7782.5 ± 563.2	8052.9 ± 422.5
Infrapyramidal blade	10010.9 ± 488.1	10974.8 ± 993.8
Middle:		
Suprapyramidal blade	8005.6 ± 552.6	8841.8 ± 991.6
Infrapyramidal blade	10670.8 ± 544.1	10901.3 ± 1249.1
Temporal:		
Suprapyramidal blade	9143.8 ± 261.2	9659.8 ± 436.4
Infrapyramidal blade	10670.0 ± 852.8	1119.9 ± 1149.7

[1]Values represent mean + S.E.M. These data were subjected to two tailed Student's t tests. No significant differences were detected.

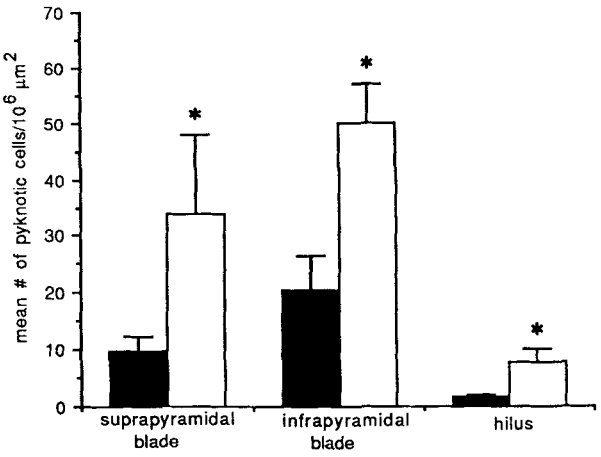

Fig. 6. The density of pyknotic cells in the rostral dentate gyrus of sham-operated (solid bar) and adrenalectomized (open bar) rat pups. Bars represent mean + SEM each obtained from at least four brains. Asterisks represent significant difference from sham ($p < 0.05$).

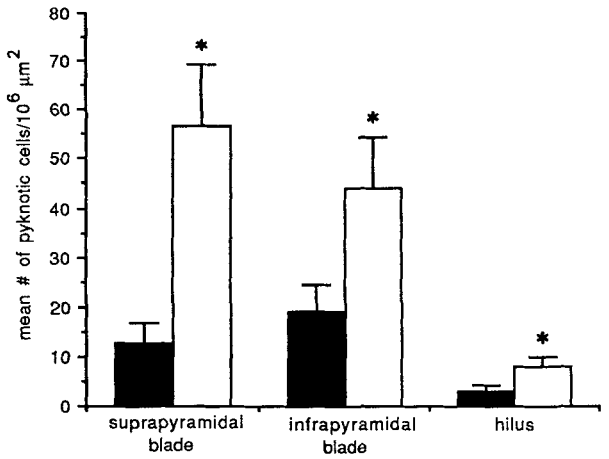

Fig. 7. The density of pyknotic cells in the middle dentate gyrus of sham-operated (solid bar) and adrenalectomized (open bar) rat pups. Bars represent mean + SEM each obtained from at least four brains. Asterisks represent significant difference from sham ($p < 0.05$).

alter the density of degenerating cells by directly affecting granule cell survival. In the adult, adrenalectomy results in massive death of granule cells; this degeneration can be prevented completely by replacing glucocorticoids (Sloviter et al., '89; Gould et al., '90). Thus, it is possible that corticosterone treatment resulted in a decrease in the density of pyknotic cells by enhancing the survival of granule cells. Such an explanation would also account for the increase in pyknotic cell density observed with postnatal adrenalectomy. However, the lack of a significant change in the density of healthy cells with either corticosterone treatment or adrenalectomy suggests that granule cell survival can not be the only process affected by glucocorticoids.

Second, glucocorticoids may regulate the migration of granule cells from their birthplace in the hilus to their final positions in the granule cell layer. Although no direct evidence is available to support the contention that low levels of glucocorticoids during the stress hyporesponsive period enable neuronal migration, some evidence is consistent with this possibility: (1) the vast majority of granule cell migration occurs during the stress hyporesponsive period (when glucocorticoid levels are low) (Rickmann et al., '87), and (2) in the adult, adrenalectomy results in an increase in the density of radial glia (unpublished observations), which presumably guide migrating granule cells in

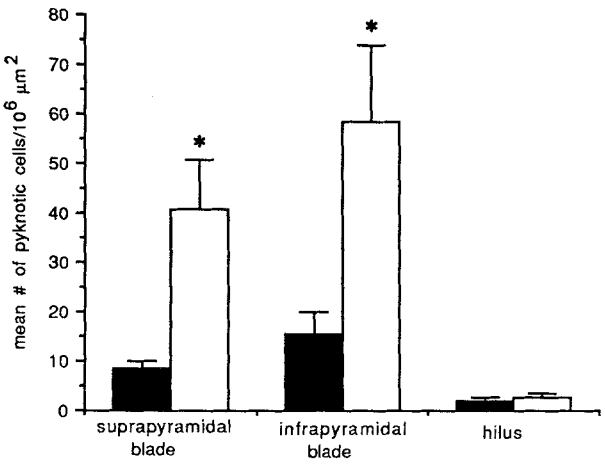

Fig. 8. The density of pyknotic cells in the temporal dentate gyrus of sham-operated (solid bar) and adrenalectomized (open bar) rat pups. Bars represent mean + SEM each obtained from at least four brains. Asterisks represent significant difference from sham ($p < 0.05$).

TABLE 4. Effects of Adrenalectomy at P15 on the density of Healthy Cells in the Dentate Gyrus[1]

Region	Sham (no. healthy cells/10^6 μm^2)	Adrenalectomy (no. healthy cells/10^6 μm^2)
Rostral:		
Suprapyramidal blade	7678.5 ± 424.3	7315.8 ± 878.2
Infrapyramidal blade	8834.0 ± 835.7	9978.5 ± 978.8
Middle:		
Suprapyramidal blade	8955.9 ± 270.1	8676.0 ± 419.8
Infrapyramidal blade	8797.3 ± 648.4	8714.0 ± 695.0
Temporal:		
Suprapyramidal blade	8783.6 ± 419.5	8665.2 ± 551.4
Infrapyramidal blade	9667.7 ± 586.5	9718.6 ± 1098.4

[1]Values represent mean ± S.E.M. These data were subjected to two tailed Student's t tests. No significant differences were detected.

the dentate gyrus. If low glucocorticoid levels are necessary for neuronal migration, it follows that excess corticosterone administered during the stress hyporesponsive period might interfere with this process, preventing newly born granule cells from reaching their appropriate destinations. This hypothesis could account for the decrease in pyknotic cell density in the granule cell layer as well as the increase in pyknotic cell density in the hilus of corticosterone-treated P6 pups compared to controls. If granule cells, newly born in the hilus, were prevented from migrating and obtaining the necessary trophic factors for their survival, then it is likely that more cells would die in close proximity to their birthplace, i.e., in the hilus. However, the hypothesis that corticosterone solely inhibits neuronal migration predicts that fewer healthy cells would be detected within the granule cell layer; our results do not confirm this. Moreover, this hypothesis cannot account for the lack of an increase in the density of pyknotic cells in the temporal hilus, a level at which corticosterone treatment significantly decreases pyknotic cell density throughout the granule cell layer. Although neuronal migration may be regulated by glucocorticoids to some extent, our results suggest that glucocorticoids probably affect a number of cellular processes in the developing dentate gyrus.

Third, glucocorticoids may influence the birth of new granule cells. Bohn ('80) has shown that administration of hydrocortisone acetate significantly decreases the density of ^{3}H-thymidine-labelled granule cells during the first postnatal week in the rat. Since we found a decrease in pyknotic cells but no change in healthy cells with corticosterone treatment, it is likely that the decrease in cell death was offset by a decrease in cell birth. This explanation could also account for the lack of a change in the density of healthy cells despite the massive increase in pyknotic cells observed with adrenalectomy. If glucocorticoids normally inhibit neurogenesis, then adrenalectomy would be expected to stimulate cell birth. Thus, the number of healthy cells in the granule cell layer remains fixed despite manipulations in glucocorticoid levels. The following paper addresses this issue and provides further evidence that neurogenesis is inhibited by adrenal steroids.

Regional differences in sensitivity to glucocorticoids

In the rat dentate gyrus, postnatal neurogenesis occurs along a general caudal to rostral gradient (Schlessinger et al., '75; Bayer, '80). Since different rostrocaudal levels of the dentate gyrus show differing degrees of maturity during the first postnatal week, it is likely that regional differences in the sensitivity to developmental factors would exist. The results of this study show that postnatal treatment with corticosterone has no effect on pyknotic cell density in the dentate gyrus at any level by P4, but significantly affects this variable at the middle and temporal levels by P6. It is possible that no glucocorticoid effects were noted in the rostral part of the dentate gyrus at P6 because these cells do not express glucocorticoid receptors during the time of hormone administration, between P2 and P6. On the other hand, removal of circulating adrenal steroids by adrenalectomy resulted, after seven days, in a dramatic rise in the density of pyknotic cells at all levels of the dentate gyrus. Although previous studies have shown that both Type 1 and Type 2 glucocorticoid receptors exist in the dentate gyrus as early as P2 (Rosenfeld et al., '88, '89), no study has yet examined the developmental expression of glucocorticoid receptors at the cellular level within specific regions of the dentate gyrus. It is possible that this uniform response to alterations in glucocorticoid levels occurs only after the entire dentate gyrus reaches a certain stage of maturity. The extent to which the results of this report correspond with the development of adrenal steroid receptors in dentate gyrus granule cells remains to be determined.

Other possible mechanisms underlying granule cell death in the developing dentate gyrus

The results of this report show that, while postnatal manipulations of glucocorticoid levels influence the density of degenerating cells in the dentate gyrus, the granule cell death which characterizes the period between P4 and P6 in the normal rat is not solely determined by low levels of glucocorticoids. Our results suggest that other factors are involved in regulation of cell death in the developing dentate gyrus. Previous studies have suggested that cell death can be controlled by the availability of trophic factors derived from target tissue (see Cowan et al., '84; Clarke, '85, for review). It is presently unknown whether granule cells which undergo pyknosis extend an axon into their target site and/or form synapses prior to their death. Studies performed in other systems, however, have shown that axon formation and target contact usually precede cell death (see Cowan et al., '84 for review). If this was also the

case for dentate gyrus granule cells, it is possible that granule cells require an as yet unidentified factor obtained from their target neurons, the CA3 pyramidal cells.

Alternatively, several studies have suggested that afferent input mediates cell death in some systems (Cunningham, '82; Clarke, '85; Furber et al., '87). Many studies performed in vitro have shown that morphology and survival of hippocampal neurons can be dramatically altered by the presence of specific neurotransmitters (see Mattson '88 for review). Interestingly, axons from the entorhinal cortex appear to invade the dentate gyrus and form synapses on granule cell dendrites during the first postnatal week (Crain et al., '73; Loy et al., '77), the period of maximal granule cell death (compare with Gould et al., '91). Input from perforant path axons involves activation of NMDA receptors on granule cell dendrites (Errington et al., '87; Ulas et al., '90). In vitro studies have demonstrated that blockade of NMDA receptors reduces the survival of cerebellar granule cells (Balazs et al., '88). It is possible that NMDA receptor activation, by excitatory input from the perforant path or intrahippocampal sources, is required for granule cell survival in the dentate gyrus. This hypothesis is currently under investigation in our laboratory.

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