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Effects of ACTH and aminoglutethimide on the catecholamine content and chromaffin cell morphology of the adrenal medulla of the neonatal rat

C. KENT AND K. G. PARKER

Department of Human Morphology, University of Nottingham Medical School, Nottingham, UK

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ABSTRACT

The rat adrenal medulla is immature at birth, composed of phaeochromoblasts and undifferentiated chromaffin cells, but by 7 d postnatally morphologically distinct adrenaline-storing (A) and noradrenaline-storing (NA) cells can be distinguished in the adult proportions of approximately 80–85% A and 15–20% NA cells. Glucocorticoid hormones are known to play an important role in the initial expression and maintenance of phenylethanolamine N-methyl transferase (PNMT), the enzyme characteristic of A cells. The purpose of the study was to investigate the effects of glucocorticoids on the establishment of the A and NA cell phenotype in vivo during the first postnatal week. Neonatal rats were treated from postnatal d 1 to 7 either with ACTH to increase circulating levels of glucocorticoids or with aminoglutethimide to reduce blood glucocorticoids. On postnatal d 7 the volume fractions of A and NA cells in the adrenal medulla were estimated and the amounts of stored adrenaline and noradrenaline determined by HPLC and compared with untreated controls. Adrenaline levels were increased following ACTH treatment and there was an apparent decrease after aminoglutethimide which was not statistically significant. There was cytological evidence of the effects of ACTH and aminoglutethimide on the adrenal cortex but no resultant effect on medullary cell morphology. A cells remained predominant with NA cells making up approximately 15% of chromaffin cells, suggesting that any effects of altered glucocorticoid levels were confined to a modulation of adrenaline synthesis by a morphologically unchanged chromaffin cell population. Plasticity of the A cell/NA cell phenotype was not demonstrated and it was concluded that although the rat chromaffin cell is immature at birth differentiation potential has already become restricted.

INTRODUCTION

Cells of the sympathoadrenal lineage depend on the environment provided by the adrenal gland for their differentiation along the endocrine line and the expression of phenylethanolamine N-methyl transferase (PNMT) (Unsicker, 1986), the enzyme that distinguishes adrenaline-synthesising (A) adrenal medullary cells from sympathetic neurons. Adrenal corticosteroids are required for the suppression of neuronal characteristics (Michelson & Anderson, 1992), the induction of PNMT, first expressed on embryonic day (E)17 in the rat fetus (Bohn et al. 1981), and subsequently for the maintenance of the activity of this enzyme (Wurtman & Axelrod, 1965) and its related mRNA (Jiang et al. 1989). Although they acquire the endocrine characteristics of chromaffin cells, a minority of adrenal medullary cells, the noradrenaline-storing NA cells, do not express PNMT even when mature. There is no evidence that they are exposed to different levels of adrenal glucocorticoids and the reason why this population of cells remains PNMT negative is unknown.

In view of the importance of glucocorticoids for maintaining the activity of PNMT the question of their role in the stability of the NA cell and A cell phenotypes in vivo was investigated by Coupland et al. (1984). Despite a maximal reduction of circulating

Correspondence to Dr C. Kent, Department of Human Morphology, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH, UK.

glucocorticoids in their experiments they observed no effect on the ratio of A cells to NA cells in young adult rats.

At birth medullary chromaffin cells of the rat are immature and appear to be intermediate between A and NA as demonstrated by immunocytochemistry (Verhofstad et al. 1985) and electron microscopy (Coupland & Tomlinson, 1989) but by 7 d postnatally the majority of the medullary chromaffin cells are typical A cells and the rest are recognisably NA cells. With approximately 20% NA and 80% A cells by volume the proportions are already close to those seen in the adult (Coupland & Tomlinson, 1989).

The object of the work presented here was to test whether or not plasticity in the A cell/NA cell phenotype can be demonstrated in vivo in the rat during the critical period immediately after birth, i.e. between 0.5 d postnatally, when distinct A and NA cells cannot be distinguished, and d 7 when the phenotypes are clearly identifiable by their ultrastructural characteristics. Von Dalnok & Menssen (1986) have reported that treatment with exogenous glucocorticoids in the early postnatal period shifts the chromaffin cell population towards the A cell phenotype but this was on the basis of incidental observations and not supported by quantitation. We treated neonatal rats for the 1st week of life either with a dose of ACTH known to induce increased blood levels of corticosteroid or with aminoglutethimide (AG), a substance that inhibits adrenal steroid synthesis. Effects on adrenal levels of adrenaline and noradrenaline were measured by HPLC and A cell/NA cell volume ratios were determined by performing volume fraction measurements on electron micrographs of randomly selected fields of the adrenal medulla.

MATERIALS AND METHODS

Wistar rats bred in the University of Nottingham Medical School Animal Unit were used. Mothers were fed Pilsbury 41B modified rat pellets (Heygates Ltd, Northampton) and water ad libitum and housed under a cycle of 12 h light and 12 h darkness at 21 °C.

Groups of 0.5-d-old rat pups were randomly assigned to each of 5 treatments: (1) unhandled, (2) daily intraperitoneal (i.p.) injections of 0.8 mg/kg adrenocorticotrophic hormone (ACTH) in 0.9% saline, (3) 0.5 ml 0.9% saline i.p. daily (saline control), (4) daily i.p. injections of aminoglutethimide (AG) (100 mg/kg) in 30% ethyl alcohol in water, (5) 0.05 ml 30% ethyl alcohol in water i.p. daily (alcohol control). Between treatments, pups were returned to the mother and allowed to suckle freely. On postnatal d 7 all animals were anaesthetised by an i.p. injection of sodium pentobarbitone (60 mg/kg).

Catecholamine assay

Freshly dissected adrenal glands were homogenized in 0.5 ml ice-cooled 0.1 M perchloric acid and 0.4 mM sodium metabisulphate (PCA), containing 5 nmol 3,4-dihydroxybenzylamine as an internal standard. The homogenate was centrifuged at 30000 *g* at 4 °C for 20 min and the supernatant stored at −80 °C before catecholamine analysis by high performance liquid chromatography (HPLC). For analysis the supernatant was diluted 1:100 with 0.1 M PCA and aliquots of 100 µl were injected into the HPLC system. This comprised a 5 µm ODS reverse-phase ion-pair column (Spherisorb 5 ODS2, Technicol Ltd) attached to an electrochemical detector incorporating a glassy carbon electrode (BAS Ltd) set at 1 namp/V, at a potential of 0.54 V. Mobile phase buffer comprised 12% methanol in 0.1 M-NaH_2PO_4, 1.2 mM-$CH_3(CH_2)_4SO_3Na$ and 0.1 mM-EDTA, adjusted to pH 3.6. The peak areas corresponding to adrenaline (A) and noradrenaline (NA) were integrated with reference to those of the internal standard in each sample and the final concentration of A and NA expressed in nmol per adrenal pair.

Electron microscopy and stereology

Adrenal glands were prepared from 5 animals from group 1 (unhandled control), 5 from group 2 (ACTH treated) and 5 from group 4 (AG treated). The animals were anaesthetised and fixed by cardiac perfusion with a buffered saline solution followed by 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4. Adrenal glands were weighed, cut with a razor blade into 3–4 parallel slices on an axis chosen at random, fixed for 2 h and processed as 3–4 tissue blocks for Araldite embedding. Volume fractions (Vv) of adrenal medullary tissue components, including A and NA cells, were estimated by point counting (Weibel, 1979). Electron micrographs (magnification ×4200, calibrated with a carbon grating replica) of randomly selected areas from sections cut from each of the tissue blocks derived from a single adrenal were analysed using a test grid for which the area per point was 3 cm^2 (equivalent to 17 μm^2 on the scale of the specimen). The results were analysed using Student's t test.

Table 1. *Catecholamine content of adrenal glands of 7-d-old rats assayed by HPLC*

Treatment (n)	Adrenal wt (mg)	Adrenaline (nmol/adrenal pair)	Noradrenaline (nmol/adrenal pair)
Unhandled (9)	3.5 (0.35)	8.5 (0.66)	2.1 (0.12)
ACTH (6)	4.9 (0.20)***	10.5 (0.37)*	1.7 (0.07)**
Saline vehicle (6)	3.1 (0.13)	9.6 (0.20)	2.0 (0.01)
Aminoglutethimide (8)	8.4 (0.70)***	9.9 (1.41)	3.8 (0.38)
Alcohol vehicle (8)	3.6 (0.19)	13.3 (1.69)	3.8 (0.53)

N, number of animals. Results expressed as means (S.E.M.). Differs from vehicle alone on Student's t test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; all other differences not significant.

Adrenaline content as a percentage of total catecholamines (controls): unhandled, 80; saline, 80; alcohol vehicle, 78; (treated): ACTH, 86; aminoglutethimide, 72.

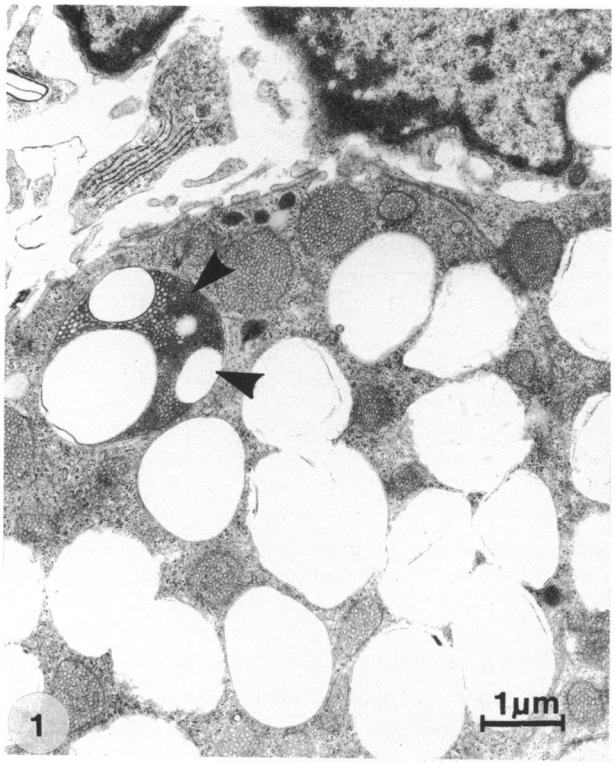

Fig. 1. Typical appearance of a cortical cell after treatment with aminoglutethimide for 7 d. Note the disrupted mitochondrion (arrowed).

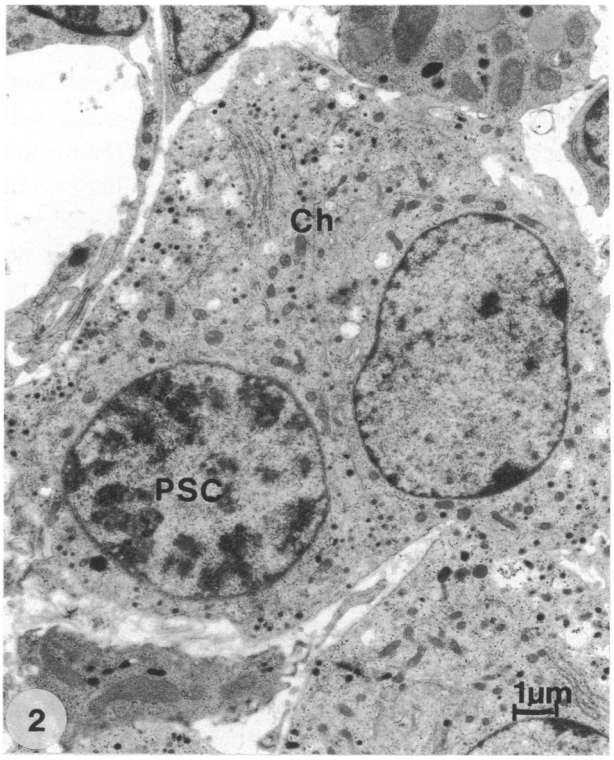

Fig. 2. Immature adrenal chromaffin cells (Ch) from unhandled rats on postnatal d 0.5. A late primitive sympathetic cell (PSC) with a rounded chromatin-rich nucleus and sparse granules is present.

RESULTS

The recipients of both drugs were less active than their litter mates and the body weights at 7 d were approximately 15% lower in the experimental group compared with the controls. Following both i.p. ACTH and AG for 7 d the weights of the adrenal glands were significantly increased, from the mean control value of 3.5 mg to 4.9 mg following ACTH and to 8.4 mg after AG (Table 1). The increase in adrenal weight after ACTH administration was due entirely to hyperplasia of the cortex with cells of normal ultrastructural appearance, whilst the administration of AG resulted in an enlarged cortex largely made up of cells seen at the fine structural level to contain abnormal amounts of cytoplasmic lipid and damaged mitochondria (Fig. 1). The volume of the medulla, judged by the inspection of large numbers of semithin sections in the course of the work, was unchanged by either of the treatments.

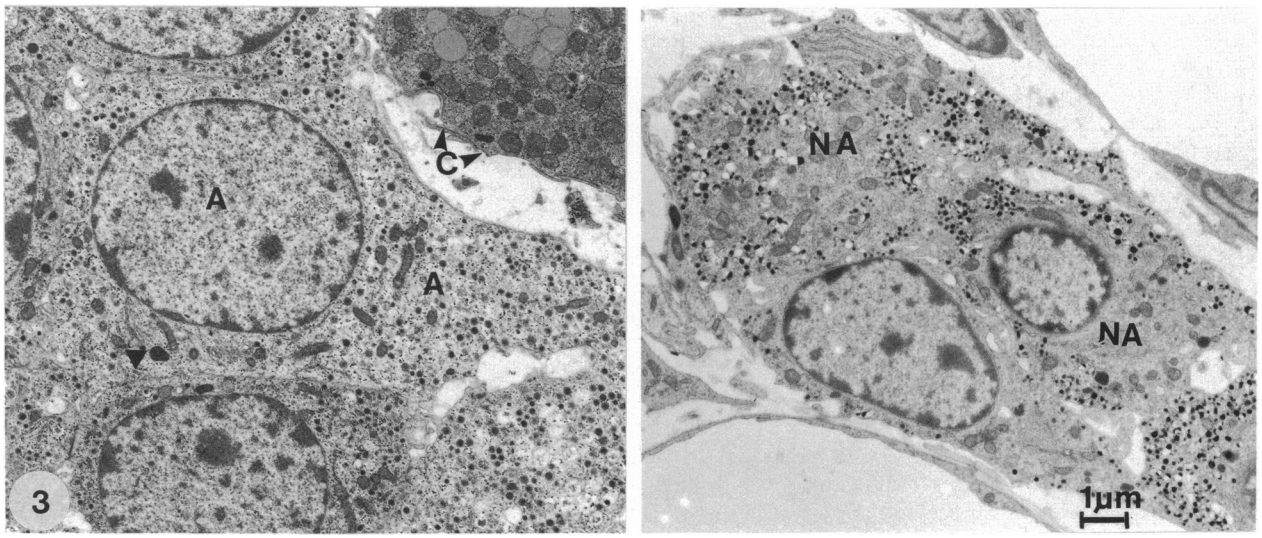

Fig. 3. Medullary chromaffin cells with typical adrenaline (A) and noradrenaline (NA)-storing granules from unhandled control rats on postnatal d 7. C, steroid-secreting cortical cell.

Table 1 shows that following 7 d of treatment with ACTH, adrenaline levels in the adrenal gland were higher and noradrenaline levels lower than those measured in the appropriate controls (saline). After AG the adrenaline content was lower compared with the alcohol vehicle control but the difference was not statistically significant. Adrenaline constituted between 72% and 86% of the total catecholamines.

The adrenal gland of control rats at 7 d had a well defined corticomedullary junction but the medulla still contained conspicuous islands of cortical cells: figures for medullary tissue components exclude cortical cells. In contrast to the indeterminate chromaffin cells seen at d 1 (Fig. 2), it was possible to characterise the chromaffin cells as either A cells, containing storage granules with a finely granular core placed concentrically within the limiting membrane, or as NA cells, with storage granules with a homogeneous, more electron-dense core often slightly eccentrically situated in relation to the membrane (Fig. 3). Small granule chromaffin (SGC) cells, recognisable by their small (110 nm diameter), dense-cored storage granules, were present as isolated cells or in small groups in all 3 groups of adrenals. The general appearance of medullary tissue from neonates treated with either ACTH or AG was similar to that of the controls except that in both instances the corticomedullary junction was less distinct and the interstitial tissue appeared more extensive.

The only effect the treatments had on the tissue components of the neonatal adrenal medulla, detected by the volume fraction measurements (Table 2), was a significant decrease in the proportion of the volume taken up by blood vessels, mainly thin-walled sinusoids, and a reduction in the proportional amount of nervous tissue brought about by AG administration. The proportion of chromaffin cells identified as adrenaline-storing remained unchanged at approximately 85% in both experimental groups (Figs 4, 5) and typical NA cells were observed at the end of 7 d in all the neonates subjected to ACTH treatment (Fig. 5).

Table 2. *Volume fractions, expressed as percentages, for the tissue components of the adrenal medulla in 7-d-old rats*

	Treatment		
	Control (n = 5)	ACTH (n = 5)	Aminoglutethimide (n = 5)
A cells	31.7 (3.5)	34.7 (5.0)	43.5 (8.6)
NA cells	4.7 (2.0)	3.3 (1.1)	6.3 (2.0)
SGC cells	2.4 (1.3)	1.1 (0.6)	1.8 (0.7)
Interstitial tissue	33.0 (5.0)	34.8 (2.3)	37.0 (7.4)
Vessels	24.1 (2.7)	23.1 (3.1)	9.6 (2.3)**
Nervous tissue	4.1 (0.8)	3.0 (0.5)	1.8 (0.6)*

Values expressed as means (SEM). Differs from control on Student's t test: $^{**}P < 0.01$, $^{*}P < 0.05$; all other comparisons not significant.

DISCUSSION

The results provided no evidence for plasticity in vivo of the neonatal medullary chromaffin cell phenotype. There was no switch to A cell characteristics as might be predicted if neonatal NA cells retain the ability to respond to exposure to increased levels of glucocorticoids, and no reduction in the proportion of cells

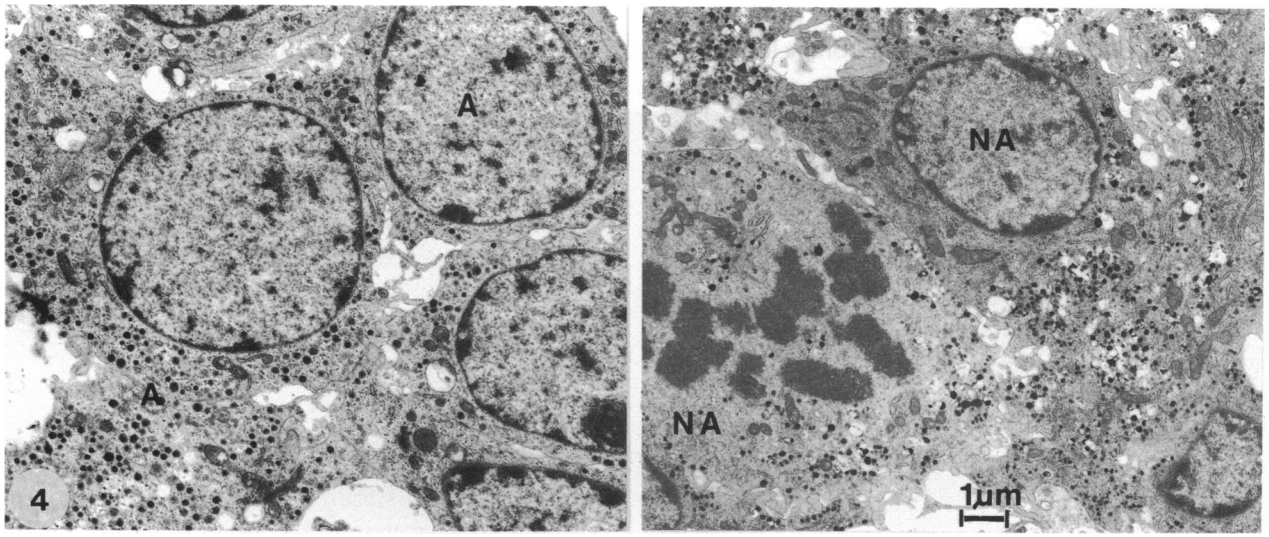

Fig. 4. Adrenaline (A) and noradrenaline (NA)-storing cells, one of which is in mitosis, on postnatal d 7 after 7 d treatment with aminoglutethimide.

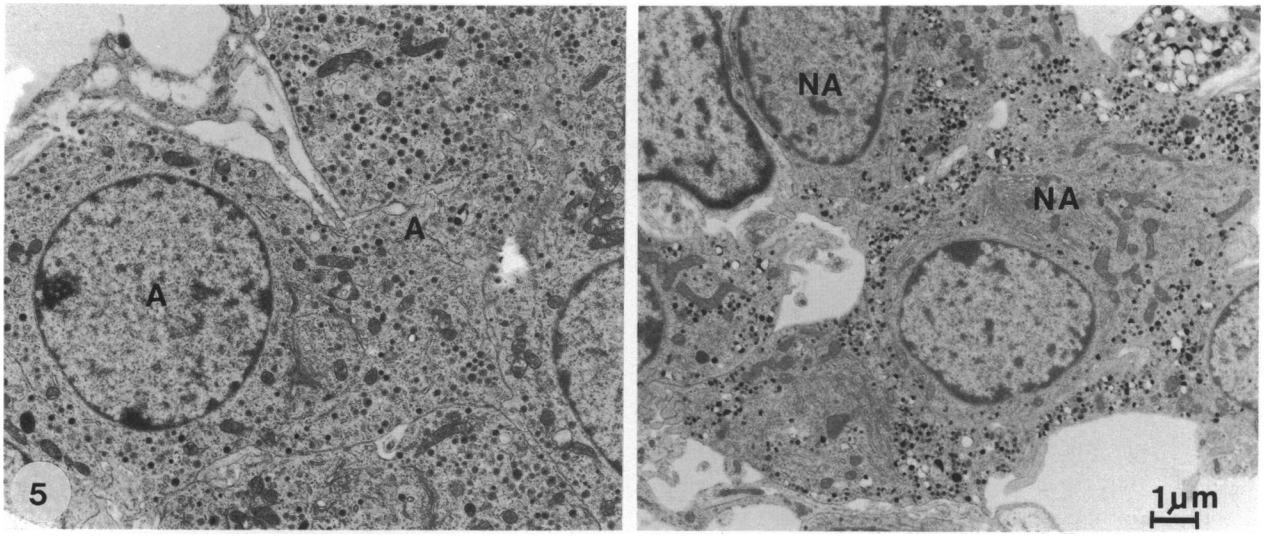

Fig. 5. Adrenaline (A) and noradrenaline(NA)-storing cells on postnatal d 7 after 7 d treatment with ACTH.

that matured into A cells when exposure to glucocorticoids was reduced by AG treatment. It is possible that the changes in circulating glucocorticoid levels were not sufficiently great to have had an effect but Banerji et al. (1986) reported 10–15 fold increases in corticosteroid levels after an identical regime of ACTH treatment during the period in question and similar doses of AG have been shown to reduce blood levels of corticosterone in rats by 80% (Moore et al. 1980). Increases in adrenal weights in the experimental groups were due by hypertrophy of the adrenal cortex and indicated a marked effect after administration of both substances. The ultrastructural appearance of the cortex after AG suggested extensive damage to corticosteroid-producing cells.

No shift in the morphological phenotype was detected by the volume fraction measurements but the increase in the amount of adrenaline stored following ACTH suggests that increased glucocorticoid levels had induced increased PNMT activity in A cells and the apparent decrease following AG treatment, although not statistically significant, indicates that decreased glucocorticoid levels might have had the reverse effect. This is in line with the finding of Coupland et al. (1984) in the young adult rat and Banerji et al. (1986) in neonatal rats. The reason for the very modest changes in adrenal catecholamine content under conditions known to affect PNMT activity profoundly is probably that storage levels are being measured and both amines are present in excess of normal requirements. Hypophysectomy in the adult rat was reported by Jiang et al. (1989) to cause a 99% fall in PNMT activity and an 83% drop in levels of PNMT mRNA, when, under identical conditions,

Coupland et al. (1984) were unable to demonstrate significant effects on catecholamine storage levels or A cell/NA cell ratio.

The unchanged proportion of A cells in the medulla after ACTH does not accord with the 8 d rat medullae 'completely occupied' by A cells after daily injections of glucocorticoids described by von Dalnok & Menssen (1986). The discrepancy may illustrate the difficulty of sampling a subpopulation of cells that is such a small proportion of the whole. Many of the randomly selected fields in the present study contained no NA cells at all but, accepting that the estimates of Vv for NA cells were less precise than those for A cells, NA cells were undoubtedly present after 7 d of ACTH treatment (Fig. 3).

One interpretation of the results is that by the time of birth in the rat the potential for chromaffin cell differentiation has become restricted and cells already embarked on the A cell lineage complete their maturation even under conditions of reduced glucocorticoids. A subpopulation of cells that has missed some crucial triggering event does not acquire the PNMT enzyme even when subjected to elevated levels of glucocorticoids and retains the NA phenotype in the adult. Immature chromaffin cells removed entirely from the influence of the cortex by transplantation to the anterior chamber of the eye (Olson, 1970) or explanted into culture (Unsicker et al. 1978) undergo a shift of phenotype towards neuronal characteristics. Transdifferentiation can be induced by a variety of growth factors but the effect is inhibited by the presence of glucocorticoids. It is not surprising, therefore, that there was no measurable change in the A cell phenotype in the present study as the levels of glucocorticoids in the microenvironment were probably sufficient to maintain normal A cell differentiation.

The explanation for the persistence of NA cells in the adrenal medulla could lie in the observation that, unlike A cells, in the adult they do not express the GC receptor (Ceccatelli et al. 1989) and during development, therefore, they do not respond to the levels of adrenal cortical glucocorticoids that are normal cues for presumptive A cells. The alternative proposition that PNMT induction is controlled by a cell-intrinsic timed process independent of the inducing signal or the appearance of the receptors, indicated by experiments on single identified precursor cells (Michelson & Anderson, 1992), is equally consistent with our findings. We have shown that the noradrenergic chromaffin cell phenotype is resistant to exposure to elevated levels of glucocorticoids even during the neonatal period of maximum plasticity. The reason why a minority of adrenal chromaffin cells remain PNMT negative into adulthood is still unknown (Unsicker, 1993).

We conclude that, in the first week of life during which the chromaffin cell phenotypes are being established the proportion of NA and A cells in the adrenal medulla of neonatal rats is not changed by altering the levels of circulating glucocorticoid hormones by the administration of large amounts of exogenous ACTH or aminoglutethimide.

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