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Analysis of mice carrying targeted mutations of the glucocorticoid receptor gene argues against an essential role of glucocorticoid signalling for generating adrenal chromaffin cells

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SUMMARY

Molecular mechanisms underlying the generation of distinct cell phenotypes is a key issue in developmental biology. A major paradigm of determination of neural cell fate concerns the development of sympathetic neurones and neuroendocrine chromaffin cells from a common sympathoadrenal (SA) progenitor cell. Two decades of in vitro experiments have suggested an essential role of glucocorticoid receptor (GR)-mediated signalling in generating chromaffin cells. Targeted mutation of the *GR* should consequently abolish chromaffin cells. The present analysis of mice lacking GR gene product demonstrates that animals have normal numbers of adrenal chromaffin cells. Moreover, there are no differences in terms of apoptosis and proliferation or in expression of several markers (e.g. GAP43, acetylcholinesterase, adhesion molecule L1) of chromaffin cells in *GR*-deficient and wild-type mice. However, *GR* mutant mice lack the adrenaline-synthesizing enzyme PNMT and secretogranin II. Chromaffin cells of *GR*-deficient mice exhibit the typical ultrastructural features of this cell phenotype, including the large chromaffin granules that distinguish them from sympathetic neurones. Peripherin, an intermediate filament of sympathetic neurones, is undetectable in chromaffin cells of *GR* mutants. Finally, when stimulated with nerve growth factor in vitro, identical proportions of chromaffin cells from *GR*-deficient and wild-type mice extend neuritic processes. We conclude that important phenotypic features of chromaffin cells that distinguish them from sympathetic neurones develop normally in the absence of *GR*-mediated signalling. Most importantly, chromaffin cells in *GR*-deficient mice do not convert to a neuronal phenotype. These data strongly suggest that the dogma of an essential role of glucocorticoid signalling for the development of chromaffin cells must be abandoned.

Key words: Sympathoadrenal cell lineage, Glucocorticoid signalling, Chromaffin phenotype, Mouse

INTRODUCTION

The neural crest and its derivatives have long occupied and continue to play a paradigmatic role in studies designed to unravel molecular mechanisms that underlie the specification of neural cell fates. The sympathoadrenal (SA) cell lineage is a major derivative of the neural crest, which gives rise to sympathetic neurones, adrenal and extra-adrenal chromaffin cells, and small intensely fluorescent cells of sympathetic and paraganglia (Landis and Patterson, 1981; Anderson, 1993; Unsicker, 1993). An essential role in triggering chromaffin as opposed to sympathetic neuronal fate has been attributed to glucocorticoid hormones (Unsicker et al., 1978; Doupe et al., 1985; Anderson and Axel, 1986; Anderson and Michelsohn, 1989; Michelsohn and Anderson, 1992). Evidence based on in vitro studies with mammalian SA progenitors has suggested that glucocorticoids are necessary for two important sequential steps in chromaffin cell development: first, to suppress neuronal markers in SA progenitors channelling them towards a chromaffin cell phenotype and, second, to induce the adrenaline-synthesizing enzyme, PNMT, in a subpopulation of chromaffin cells (Bohn et al., 1981; Anderson and Axel, 1986;. Anderson and Michelsohn, 1989; Michelsohn and Anderson, 1992). Moreover, the ability of nerve growth factor (NGF) and other growth factors to induce a neuronal phenotype in young chromaffin cells, and the capacity of glucocorticoids to prevent

this shift in phenotype has further helped to assume an essential role of glucocorticoid hormones in chromaffin cell development (Unsicker et al., 1978, 1984; Seidl and Unsicker, 1989a,b; Doupe et al., 1985).

Inactivation of the *GR* gene is incompatible with survival. Mice die at birth due to lung failure (Cole et al., 1995; Tronche et al., 1998; F. T., C. K., and G. S., unpublished). This precludes any analysis of the function of *GR* in adult animals. However, we analysed the function of *GR* in adrenal development. Contrary to expectations based on two decades of in vitro experiments with SA progenitor and chromaffin cells, mice deficient in GR-mediated signalling have normal numbers of adrenal chromaffin cells. As expected, *GR*-deficient mice do not express PNMT, the adrenaline-synthesizing enzyme of chromaffin cells, which has long been known to be under strict control of glucocorticoid hormones in mammals, but not in many submammalian vertebrates (Wurtman and Axelrod, 1965; Wurtman et al., 1968). Most importantly, chromaffin cells of *GR*-deficient mice do not adopt a neuronal phenotype and do not express features typical of sympathetic neurones, e.g. neuronal intermediate filaments and a massive neurite outgrowth response to nerve growth factor (NGF). In summary, our data contradict the established idea of an essential role of glucocorticoid signalling in chromaffin cell development.

MATERIALS AND METHODS

Antibodies and reagents for immunocytochemistry

Commercial antibodies and immunoreagents were obtained from the following sources (dilution and required references in brackets): polyclonal sheep anti-tyrosine hydroxylase (TH, 1:100) and polyclonal rabbit anti-peripherin antibodies (1:100, Chemicon International, Temecula, CA); polyclonal rabbit anti-phenylethanolamine-N-methyl-transferase (PNMT, 1:1000, Incstar, Stillwater, OK); monoclonal rat anti-Growth Associated Protein-43 (GAP-43, 1:500, Grant et al., 1994; Sigma Immunochemicals, St Louis, MO); monoclonal mouse anti-Proliferating Cell Nuclear Antigen (PCNA, 1:500, Novocastra Laboratories, Newcastle, UK). Normal goat serum, normal rabbit serum and normal horse serum were from Vector Laboratories, Burlingame, CA. Biotinylated goat anti-rabbit, biotinylated horse anti-mouse, biotinylated rabbit anti-sheep IgG antibodies, Vectastain ABC-AP kit, alkaline phosphatase substrate kit, and Elite ABC-kit were also purchased from Vector Laboratories. Cy3™, FITC™ and AMCA-conjugated streptavidin were from Jackson Immuno Research Laboratories, Inc., Westgrove, PA; ApoTag™ In Situ APOPTOSIS Detection Kit was from Oncor, Gaithesburg, MD.

The following antibodies were obtained from non-commercial sources: anti-acetylcholinesterase (1:500, Dr Massoulié, Laboratoire de Neurobiologie, École Normale Supérieure CNRS URA 1857, Paris); antibodies to chromogranin B (1:500) and secretogranin II (1:200, Rosa et al., 1985; Dr Huttner, Institute of Neurobiology, Heidelberg); antibodies against the vesicular catecholamine transporters VMAT-1 (endocrine) and VMAT-2 (neuronal; Dr Hannah, Institute of Neurobiology, Heidelberg); antibodies to the transcription factor Phox-2a (1:500, Tiveron et al., 1996; Drs Jean Francois Brunet and Christo Goridis, IBDM, Marseille, France); antibodies to neuropeptides NPY (1:500, Garcia-Arraras et al., 1992); somatostatin (1:3000, Garcia-Arraras et al., 1984); met-enkephalin (1:500, Dr Barreto-Estrada); galanin (GAL; Diaz-Miranda et al., 1996) and L1 (Dr Faissner, Centre de Neurochimie, Strasbourg, France).

Gene targeting strategies

Two distinct mutations in the *GR* gene were used in this study. The $GR^{hypo/hypo}$ mice were described previously (Cole et al., 1995). They were generated by disrupting the mouse *GR* gene in ES cells using an insertion vector strategy. Briefly, a selectable marker, the neomycin phosphotransferase gene cassette (PGKNEO), was inserted into exon 2 of the mouse *GR* gene. Mice heterozygotes generated as previously described were intercrossed to generate the $GR^{hypo/hypo}$ mice.

Although most $GR^{hypo/hypo}$ mice died at birth, about 20% survived suggesting that inactivation of the *GR* gene might have not been complete in these mutants. Therefore, a new mutant obtained by deletion of the third exon ensuring complete inactivation has been generated. The strategy followed to obtain the GR^{null} allele will be described elsewhere. Briefly, a gene targeting vector was constructed from 129 mouse genomic DNA, in order to flank the third *GR* exon by two loxP sites, following the strategy described by Gu et al. (1994). The two loxP sites are inserted 1.9 kb apart. We obtained modified E14-1 embryonic stem cells (Kühn et al. 1991) that had inserted in their genome the modified sequence. Transient transfection with a Cre recombinase expression vector led to the deletion of the DNA sequence located between the two loxP sites, therefore generating the GR^{null} allele in which the third exon is deleted. Cells from ES subclones were injected into blastocysts to derive $GR^{null/+}$ animals. Heterozygous mice were intercrossed.

Histology

Embryos were recovered, rinsed in cold phosphate-buffered saline (PBS), fixed in PBS containing 4% paraformaldehyde (PFA), for 4-8 hours, depending on the developmental age and antibodies used. In some instances, when indicated, embryos at the developmental age E18.5 were perfused through the left ventricle of the heart with PFA. Embryos and tissues of interest were then rinsed in PBS and placed in 10% sucrose followed by immersion in 30% sucrose in PBS at 4°C, coated in OCT compound and kept frozen at −70°C until used. Cryostat sections were serially cut at 10 μm and placed on gelatin/chrome-alum-treated slides, dried at room temperature for 30 minutes, immersed in 0.1 M sodium phosphate (PB, pH 7.4) and then processed for immunohistochemistry. For visualizing apoptotic and proliferating cells, respectively, whole embryos were taken at E13.5 and E15.5, fixed in PFA overnight, dehydrated through increasing concentrations of ethanol before embedding in paraffin wax. Serial sections (7 μm) were placed on silan-coated slides, dried at 37°C and kept at room temperature.

TdT dUTP nick end labeling (TUNEL) analysis

For detection of apoptotic adrenal chromaffin progenitor cells, TUNEL was performed according to the manufacturer's instructions and as previously described (Finotto et al., 1997). Three mice for each group and 1,000 TH-positive cells per mouse were counted after staining using ×400 magnification. Results are expressed as a percentage of apoptotic TH-positive chromaffin cells.

Immunocytochemistry

For monitoring cell proliferation, we used an antibody directed against the proliferating cell nuclear antigen (PCNA), which detects the delta DNA polymerase accessory protein (Bravo et al., 1987; Waseem et al., 1990). Tissue sections were dewaxed through xylene, rehydrated through a graded series of ethanols and washed in PBS. Endogenous peroxidase was inhibited with 3% hydrogen peroxide in methanol for 10 minutes. Slides were then incubated with an anti-mouse PCNA antibody (1:100) in PBS with 0.1% BSA for 1 hour at room temperature. Sections were then washed with PBS and incubated with a biotinylated horse anti-mouse antibody (1:100) followed by avidin and biotinylated horseradish-peroxidase-macromolecular complex (Vector: Elite ABC reagent) and DAB according to the manufacturer's instructions. In order to evaluate the number of proliferating chromaffin cells, tissues were then stained with an antibody to TH as

described above. Three mice for each group and 1,500 TH-positive adrenal cells were counted at ×400 magnification.

For immunocytochemistry using neuropeptides, enzymes and other antigens, 10 μm thick sections were pretreated with 10% serum corresponding to the secondary antibody, in PBS and 0.1% Triton X-100 to inhibit non-specific binding, followed by incubation with the primary antibody overnight at 4°C. The following day sections were rinsed in PBS and incubated with a biotinylated secondary IgG antibody (1:100) for 1 hour at room temperature followed by incubation with a Cy3™-conjugated strepatavidin (Dianova, Hamburg, Germany; 1:500) for 1 hour at room temperature. Sections were rinsed with PBS, mounted with glycerol gelatin (Merck, Darmstadt, Germany) and observed in a Zeiss Axiophot microscope. Three mice per group and at least 9 sections per adrenal gland at its largest diameter including the medulla were immunostained and subsequently analyzed. Where indicated, whole adrenal glands were serially sectioned.

Chromaffin cell cultures

Embryos at E17.5 were removed from the uterine horns under sterile conditions. Adrenal glands were dissected and separately processed as previously described (Unsicker et al., 1989). Cell suspensions were seeded at a density of 1,000 cells per cm^2 on polyornithine/laminin-coated plastic surfaces or glass coverslips in DMEM and the N1 supplements (cf. Unsicker et al., 1989) with or without NGF (Boehringer/Mannheim; 50 ng/ml). After 96 hours, coverslips were removed, fixed in 4% PFA for 10 minutes followed by postfixation in cold acetone for 5 minutes and subject to TH immunocytochemistry as described above. Neurites of TH-positive cells were scored if more than 2-fold longer than the somal diameter. Adrenal glands from three mice per group ($GR^{null/null}$, wild type) were analyzed.

Electron microscopy

For electron microscopy, adrenals from E14.5 and E16.5 embryos were fixed by immersion in a mixture of glutaraldehyde (1.5%) and paraformaldehyde (1.5%) in phosphate buffer at pH 7.3 for 48 hours and then rinsed thoroughly in several changes of cacodylate buffer (0.1 M). E18.5 embryos were perfused through the left ventricle of the heart with a mixture of glutaraldehyde (1.5%), paraformaldehyde (1.5%) and polyvinylpyrrolidone (PVP; 2.5%) in phosphate buffer at pH 7.3. Organs from all embryonic ages were then postfixed in 1% OsO_4/1.5% potassium hexanoferrate, rinsed in 0.1 M cacodylate buffer and 0.2 M sodium malate buffer (pH 6.0) and block-stained with 1% uranyl acetate. Semithin sections (1 μm) were stained with toluidine blue. Ultrathin sections (50 nm) were examined with a Zeiss EM10.

Morphometry

Ultrathin sections were cut from Epon-embedded adrenal gland anlagen from E14.5, E16.5 and E18.5 wild type, $GR^{hypo/hypo}$ and $GR^{null/null}$ (3 adrenal glands per group). Chromaffin granules were measured on electron micrographs from randomly selected sections (n=14 per adrenal gland) at a final magnification of ×15,550 using an automatic image analysis system (Quantimet 500C, Leica, Bensheim, FRG). Determined parameters were volume density and diameter of granules (dense cores).

Statistical analysis

Data were analyzed by a one-way ANOVA, and the significance of intergroup differences were determined by applying Student's t-test (Microcal Origin V. 3.54). Differences were considered significant at $*P<0.05$, $**P<0.01$, $***P<0.001$.

RESULTS

Development of gene disruption technology in mice allows in vivo hypotheses resulting from cell culture approaches to be challenged. Disruption of the *GR*, neurotrophins and neurotrophin receptors (cf. Schober et al., 1997, 1998) furnish ideal tools to analyse the role of glucocorticoids and neurotrophins in the development of chromaffin cells and their innervation. Two different mutations have been generated in the *GR* gene. The first was made by the insertion of a DNA cassette into the second GR exon (GR^{hypo} allele Cole et al., 1995; Tronche et al., 1998). This modification resulted in a *GR* allele that generates mRNA species containing an ORF encoding an amino-terminal truncated protein with intact DNA and ligand-binding domains (H. Reichardt, personal communication). Although most $GR^{hypo/hypo}$ mice died at birth, about 20% survived to adulthood suggesting that inactivation of the GR gene might not have been complete in these mutants. Confronted with this result, we generated an independent GR mutant allele in mice (GR^{null} allele; Fig. 1A). To ensure a complete inactivation of GR function, the third exon, which encodes the first zinc-finger of the DNA-binding domain, was deleted (Fig. 1B). This deletion led in animals to a more pronounced phenotype, including the fact that 100% of $GR^{null/null}$ mice died shortly after birth (F. T., C. K., G. S. unpublished data).; no $GR^{null/null}$ mice were present among 286 descendants of $GR^{null/+}$ intercrosses genotyped at 4 weeks of age while they occurred with a Mendelian distribution in litters obtained by Cesarian section. Due to this difference in $GR^{hypo/hypo}$ and $GR^{null/null}$ phenotypes, we decided to study adrenal development in parallel within these two families.

Numbers of TH- and Phox-2-positive adrenal chromaffin cells are not affected by the *GR* knockouts

Histological analysis of adrenal glands from $GR^{hypo/hypo}$ mice had shown (Cole et al., 1995) the lack of a solid adrenal medulla

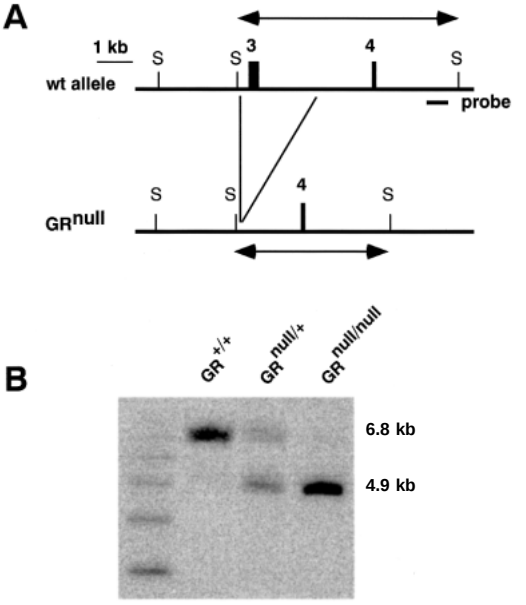

Fig. 1. Inactivation of the *GR* gene. (A) Schematic diagram of the wild-type *GR* allele and GR^{null} allele. The third and fourth exons are indicated by black boxes. (B) Southern blot analysis of tail DNA of progeny from heterozygote intercrosses. DNA was digested with *Sac*I and probed with the DNA fragment indicated. Filters were exposed overnight.

and suggested a reduced number of TH-positive cells as compared to wild-type littermates. TH expression is known to be controlled by glucocorticoids (Lewis et al., 1987) and might be expected to be downregulated in *GR*-deficient mice giving false negative results when TH is used as a marker for chromaffin cells. We therefore used, in addition to TH, another independent marker, the transcription factor Phox-2, for monitoring chromaffin cells. Phox-2 has been shown to be expressed by all peripheral catecholaminergic cells (Tiveron et al., 1996; Groves et al., 1995). Using serial sections of wild-type adrenal glands of different embryonic ages, we ascertained that TH and Phox-2 are strictly co-localized in chromaffin cells at E13.5 and E18.5, respectively (not shown). Consistent with this notion, cell counts revealed small, yet insignificant discrepancies in the numbers of TH- and Phox-2-positive cells in E13.5 and E18.5 adrenal glands (Fig. 2). Comparisons of wild-type and $GR^{hypo/hypo}$ mice did not reveal significant differences in the numbers of TH- and Phox-2 cells at E13.5 and E18.5 (Fig. 2). Moreover, cell counts performed at E18.5 with $GR^{null/null}$ mice confirmed that there were no significant losses of chromaffin cells in either of the two GR mutants (Fig. 2).

Apoptosis and proliferation of developing adrenal chromaffin cells are not increased in *GR*-deficient mice

To further ensure that chromaffin cells of *GR*-deficient mice undergo apoptosis and proliferation to the same extent as wild-type littermates, we used the TUNEL technique and staining for PCNA, respectively. Serial sections of adrenal glands from E13.5 and E15.5 $GR^{hypo/hypo}$ and wild-type mice were analyzed by double staining with an antibody to TH and the TUNEL technique. As shown in Fig. 3A, numbers of apoptotic TH-positive cells were not significantly different at either time point.

Embryonic chromaffin cells continue to divide following their migration into the adrenal gland (Verhofstad, 1993; Tischler et al., 1989, 1991). Co-staining with antibodies to TH and PCNA revealed that adrenal chromaffin cell proliferation did not significantly differ in wild-type and $GR^{hypo/hypo}$ mice at E13.5 and E15.5 (Fig. 3B). In confirmation of the cell count data shown above, similar results with regard to apoptosis and proliferation of TH-positive cells were obtained with $GR^{null/null}$ mice (not shown).

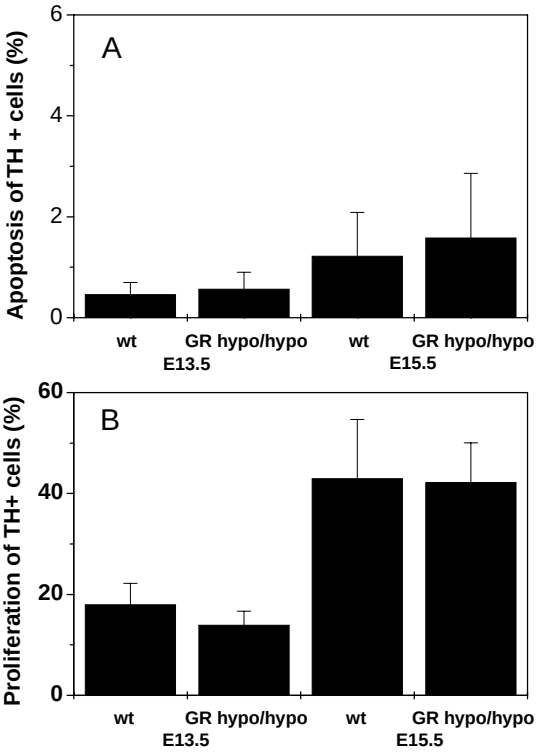

Fig. 3. Apoptosis and proliferation of TH+ adrenal chromaffin cells during embryonic development in wild-type and $GR^{hypo/hypo}$ mice. (A) Quantification of TUNEL-positive TH-immunoreactive adrenal chromaffin cells. There is no significant difference between wt and $GR^{hypo/hypo}$ mice in the number of chromaffin cells undergoing apoptosis at E13.5 and at E15.5. Data are presented as mean ± s.e.m. (n=3). Student's t-test (two tailed) was used to compare groups. (B) Proliferation of TH+ adrenal chromaffin cells in wild-type and $GR^{hypo/hypo}$ mice at E13.5 and E15.5, using PCNA as a marker of proliferation. Data are presented as mean ± s.e.m. No statistical difference was observed between the two groups.

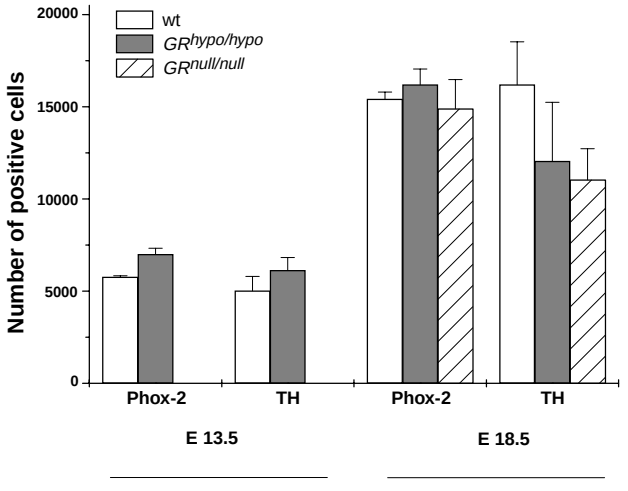

Fig. 2. Cell counts of Phox-/TH-immunoreactive adrenal chromaffin cells on serial sections of wild-type (wt), $GR^{hypo/hypo}$, and $GR^{null/null}$ mice at E13.5 and E18.5. At least 6 adrenal glands from 3 animals per group were analyzed.

Impaired expression of catecholamines, PNMT and granins in *GR*-deficient mice

Using HPLC and electrochemical detection, we analyzed adrenal glands from newborn $GR^{hypo/hypo}$, $GR^{null/null}$ and wild-type mice for catecholamine content. Table 1 shows that *GR*-deficient mice contain no adrenaline and have a reduced content of noradrenaline. Reduction of the noradrenaline content may reflect the slight, although not significant, reduction in numbers of TH-immunoreactive chromaffin cells in GR mutants (Fig. 2). Consistent with the lack of adrenaline, Fig. 4E,F demonstrates that PNMT immunoreactivity is undetectable in adrenal glands of $GR^{hypo/hypo}$ as well as $GR^{null/null}$ mice at E18.5. In wild-type littermates, PNMT immunoreactivity is clearly detectable at E15.5 (not shown) and found in a majority of TH-positive adrenal cells at E18.5 (Fig. 4A,D).

Table 1. Adrenal catecholamines in wild-type (+/+), *GR*$^{hypo/hypo}$, and *GR*$^{null/null}$ mice

	Adrenaline (% of NA+A)		Noradrenaline (% of wild type)	
	GR$^{hypo/hypo}$	*GR*$^{null/null}$	*GR*$^{hypo/hypo}$	*GR*$^{null/null}$
–/–	0.00±0.00 (*n*=5)	0.00±0.00 (*n*=3)	35.82±16.82 (*n*=5)	22.60±7.52 (*n*=3)
+/–	44.79±3.74 (*n*=7)	48.0±3.95 (*n*=7)	100.79±23.30 (*n*=7)	108.05±14.84 (*n*=7)
+/+	50.19±5.7 (*n*=7)	72.19±3.99 (*n*=3)	100 (*n*=7)	100 (*n*=3)

Data are mean±S.E.M.

Adrenal chromaffin cells contain within their secretory granules a family of acidic proteins, the granins, which have been implicated in the packaging of secretory molecules and their targeting towards the regulated pathway of secretion (Huttner et al., 1991; Winkler and Fischer-Colbrie, 1992; Rosa and Gerdes, 1994). Most chromaffin cells including the majority of PNMT-positive cells in E18.5 adrenal glands of wild-type mice display chromogranin B (CgB) immunoreactivity (not shown), while about one third of the chromaffin cells show secrotogranin II (Scg II) immunoreactivity (Fig. 4G). E18.*5* *GR*$^{hypo/hypo}$ as well as *GR*$^{null/null}$ mice completely lack ScgII immunoreactive cells (Fig. 4H,I). Numbers of CgB-positive cells are diminished (not shown).

Many markers of the adrenal chromaffin cell phenotype are not affected in *GR*-deficient mice

Adrenal chromaffin cells co-store within their secretory granules a large number of different neuropeptides along with catecholamines and other small molecules (Winkler, 1993), which have been implicated in the discrimination of distinct subsets of chromaffin cells. It should be noted, however, that most of these markers are not fully expressed until the postnatal period (cf. Schober et al., 1997). Neuropeptide Y, met-enkephalin, somatostatin and galanin immunoreactivities of chromaffin cells were qualitatively unaffected in *GR*-deficient mice (Table 2). Both wild-type and *GR*-deficient adrenal chromaffin cells and their preganglionic nerve fibers displayed weak staining for acetylcholinesterase (AChE) immunoreactivity at E18.5 (Table 2). Likewise, immunoreactivity for the adhesion molecule L1, a marker for a subpopulation of chromaffin cells (Leon et al., 1992), appeared unaltered.

Adrenal chromaffin cells in *GR*-deficient mice fail to express sympathetic neuronal markers

The dogma of an essential role of glucocorticoid signalling in triggering chromaffin as opposed to sympathetic neurone development implies that glucocorticoids suppress the expression of neuronal markers in SA progenitor cells (Michelsohn and Anderson, 1992). Accordingly, *GR*-deficient mice would be expected to have their chromaffin tissue replaced by cells expressing neuronal traits. We therefore investigated whether the adrenal TH- and Phox-2-positive cells expressed neuronal markers. Analysis of *GR*$^{hypo/hypo}$, *GR*$^{null/null}$ and wild-type adrenal glands revealed that there were, as expected, peripherin-immunoreactive nerve fibres representing the preganglionic innervation of chromaffin cells (Fig. 5). However, TH-positive cells were consistently found to lack peripherin immunoreactivity (Fig. 5). Likewise, we were not able to detect neurofilament immunoreactivity in chromaffin cells using the commercially available antibodies (not shown). We therefore conclude that loss of GR-mediated signalling does not foster expression of the neuron-specific intermediate filament protein peripherin in adrenal chromaffin cells.

To further substantiate this idea, we investigated the presence

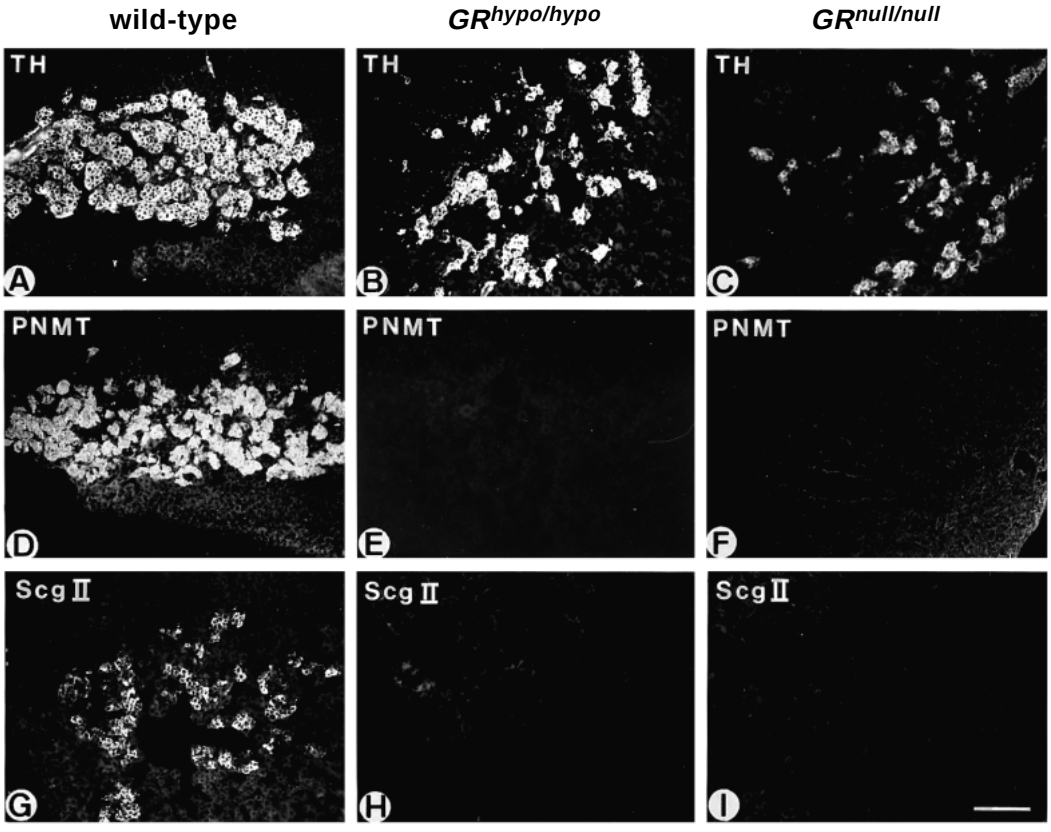

Fig. 4. Adrenal glands of E18.5 wild-type, *GR*$^{hypo/hypo}$ and *GR*$^{null/null}$ mice stained for tyrosine hydroxylase (TH), phenylethanolamine N-methyltransferase (PNMT), and secretogranin II (ScgII) immunoreactivities. Note the absence of PNMT and ScgII in *GR*-deficient mice. Bar, 100 μm.

Table 2. Immunocytochemical characterization of glucocorticoid receptor deficient mice at the developmental stage E18.5 using chromaffin associated markers

	Structure labelled	Wild-type	GR–/–
Neuropeptides			
NPY	Most chromaffin cells	+++	+++
Somatostatin	Few, small cells within the medulla, SIF-like	+	+
Met-Enkephalin	Chromaffin cell subpopulation	+	+
Galanin	No labelling at this stage	–	–
Catecholamine related			
TH	All chromaffin cells	+++	+++
PNMT	Chromaffin cell subpopulation	++	++
VMAT-1 (neuroendocrine)	Most, if not all chromaffin cells	+	+
VMAT-2 (neuronal)	Chromaffin cell subpopulation	+	+
Innervation			
AChE	Nerve fibres	+	+
L1	Nerves, extracellular labelling of chromaffin cells	+	+
Vesicular			
Chromogranin B	Chromaffin cell subpopulation	++	+
Secretogranin II	Chromaffin cell subpopulation	++	–

+ Denotes the apparent degree of labelling: +++ strong, ++ moderate, + weak, – none.

and distribution of immunoreactivities for the neuronal and neuroendocrine type, respectively, of the vesicular monoamine transporter, VMAT-2 (neuronal) and VMAT-1 (neuroendocrine). It has been shown in rodents that sympathetic principal neurones express VMAT-2, whereas the neuroendocrine SIF cells in sympathetic ganglia are positive for VMAT-1. In the (adult rat) adrenal medulla, all chromaffin cells express VMAT-1 and a subpopulation, possibly the noradrenergic, PNMT-negative chromaffin cells, are immunoreactive for VMAT-2 (Weihe et al., 1994; Peter et al., 1994, 1995). In wild-type embryonic mouse adrenal glands, VMAT-2 was detectable in a few cells at E13.5, which increased in number until E18.5. In contrast, VMAT-1 immunoreactivity could not be detected prior to E18.5. Analyses of adrenal glands from E18.5 wild-type and *GR* mutant mice failed to reveal a difference in numbers of VMAT-2-positive chromaffin cells (Table 2).

GAP43 has been reported to be exclusively associated with noradrenergic chromaffin cells in the postnatal rat adrenal gland (Grant et al., 1992). We therefore investigated the possibility that adrenal chromaffin cells in *GR*-deficient mice, which lack adrenaline and are therefore exclusively of the noradrenergic type, would display a noticeable increase in GAP43 immunoreactivity. However, most, if not all, TH-positive cells in the adrenal gland of E18.5 wild-type mice were immunoreactive for GAP43, suggesting that both noradrenergic and adrenergic chromaffin cells express GAP43 in mice. Adrenal glands of *GR*-deficient mice failed to show alterations in their numbers of GAP43-positive cells (not shown).

Identical proportions of NGF-stimulated cultured adrenal chromaffin cells of *GR*-deficient and wild-type mice extend neurites

Sympathetic neurones cultured from perinatal rodents readily respond to NGF with neurite outgrowth (Unsicker and Chamley, 1977). In contrast, less than 25% of chromaffin cells extend neurites within 4 days when challenged with NGF (Unsicker et al., 1985a,b). In order to further substantiate the notion that adrenal chromaffin cells in *GR*-deficient mice had not converted to a sympathetic neuronal phenotype, responses of $GR^{null/null}$ and wild-type chromaffin cells isolated from E18.5 mice to NGF were compared. Spontaneous neuritic growth in the absence of added trophic factors after 96 hours was seen on less than 5% of cultured TH-positive chromaffin cells in both wild-type and *GR*-deficient mice. In three independent experiments (250-450 cells per experiment), addition of NGF (50 ng/ml) resulted in an increase in the proportion of neurite-bearing cells, which was not significantly different in wild-type and *GR*-deficient mice (wild type: 14±4 %, $GR^{null/null}$: 19±7; s.e.m.). Again, these data suggest that chromaffin cells from *GR*-deficient mice had not been converted to a neuronal phenotype.

Adrenal chromaffin cells of *GR*-deficient mice have all the ultrastructural features of this cell type and are ultrastructurally clearly distinct from sympathetic neurones

Analysis of ultrathin sections of adrenal chromaffin cells from wild-type and *GR*-deficient mice at E14.5, E16.5 and E18.5 failed to reveal any overt qualitative differences in their

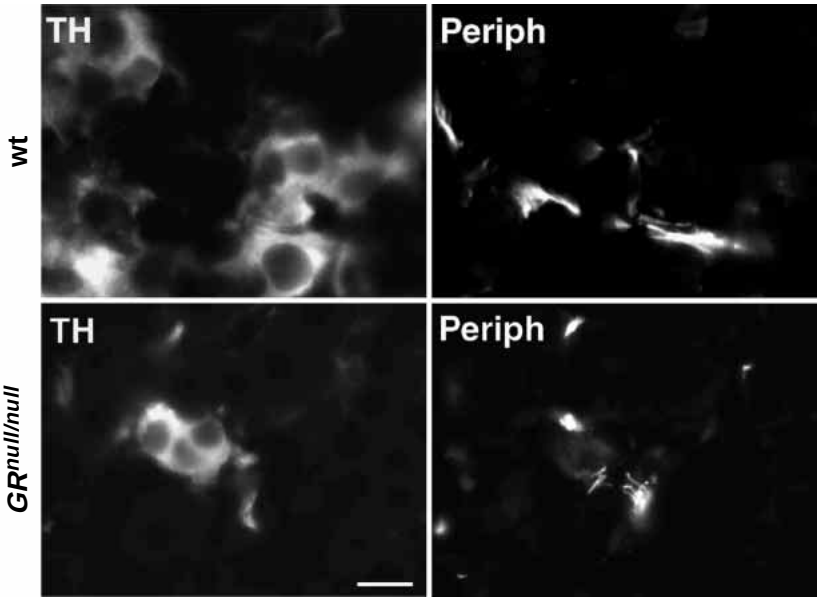

Fig. 5. Adrenal chromaffin cells in $GR^{null/null}$ mice fail to display immunoreactivity for the intermediate filament protein peripherin, a marker of sympathetic neurones. Double labellings for TH and the peripheral neuronal marker peripherin are shown in E18.5 adrenal glands of wild-type and $GR^{null/null}$ mice. Note that peripherin immunoreactivity is restricted to nerve fibres (probably of extra-adrenal origin) and absent from TH-positive cells. Bar, 15 μm.

ultrastructure (Fig. 6A-C). In particular, typical features of principal sympathetic neurones (Eränkö, L., 1972; Eränkö, O., 1972), for example, large nuclei with prominent nucleoli, a large perinuclear cytoplasmic area with well-developed cisternae of the Golgi apparatus and ribosome-studded endoplasmic reticulum, or axonal and dendritic processes were not encountered. All chromaffin cells contained large dense-cored vesicles (LDV) typical of this cell type ('chromaffin' LDV or 'chromaffin granules'). Both their number and diameter increased during embryonic development, both in $GR^{hypo/hypo}$ mice and their wild-type littermates (Fig. 7). Morphometric analysis of the parameters 'volume density' (i.e. the percentage of the cell area occupied by granules), and 'granule diameter' revealed no significant differences between wild-type and $GR^{hypo/hypo}$ mice at E18.5 (Fig. 7). The average diameter of chromaffin granules (determined as the diameter of the dense core) was 116±3.87 nm in wild-type as compared to 107.45±3.37 nm in $GR^{hypo/hypo}$ mice at E18.5, which is in good agreement with the average diameter of chromaffin granules in the newborn rat (see Discussion). However, volume density and granule diameter slightly differed between wild-type and $GR^{hypo/hypo}$ mice at E14.5 and E16.5, respectively (Fig. 7), suggesting a role of glucocorticoid signalling in the regulation of chromaffin granule number and size at early time points of adrenal chromaffin cell development. Semiquantitative evaluation of chromaffin granules in $GR^{null/null}$ mice performed on E18.5 adrenal glands likewise failed to detect apparent differences in numbers of granules per cell area and granule diameter, respectively.

DISCUSSION

Deficiency in glucocorticoid signalling does not abolish adrenal chromaffin cells or a putative chromaffin sublineage and leaves most of their phenotypic markers unimpaired

The present analyses of chromaffin cell development in $GR^{hypo/hypo}$ and $GR^{null/null}$ mice show that loss of *GR*-mediated signalling does not abolish or reduce adrenal chromaffin cell numbers. Moreover, they provide evidence for a largely unimpaired development and phenotype of *GR*-deficient chromaffin cells. Numbers of chromaffin cells monitored by staining for TH and the transcription factor Phox-2, a specific marker of

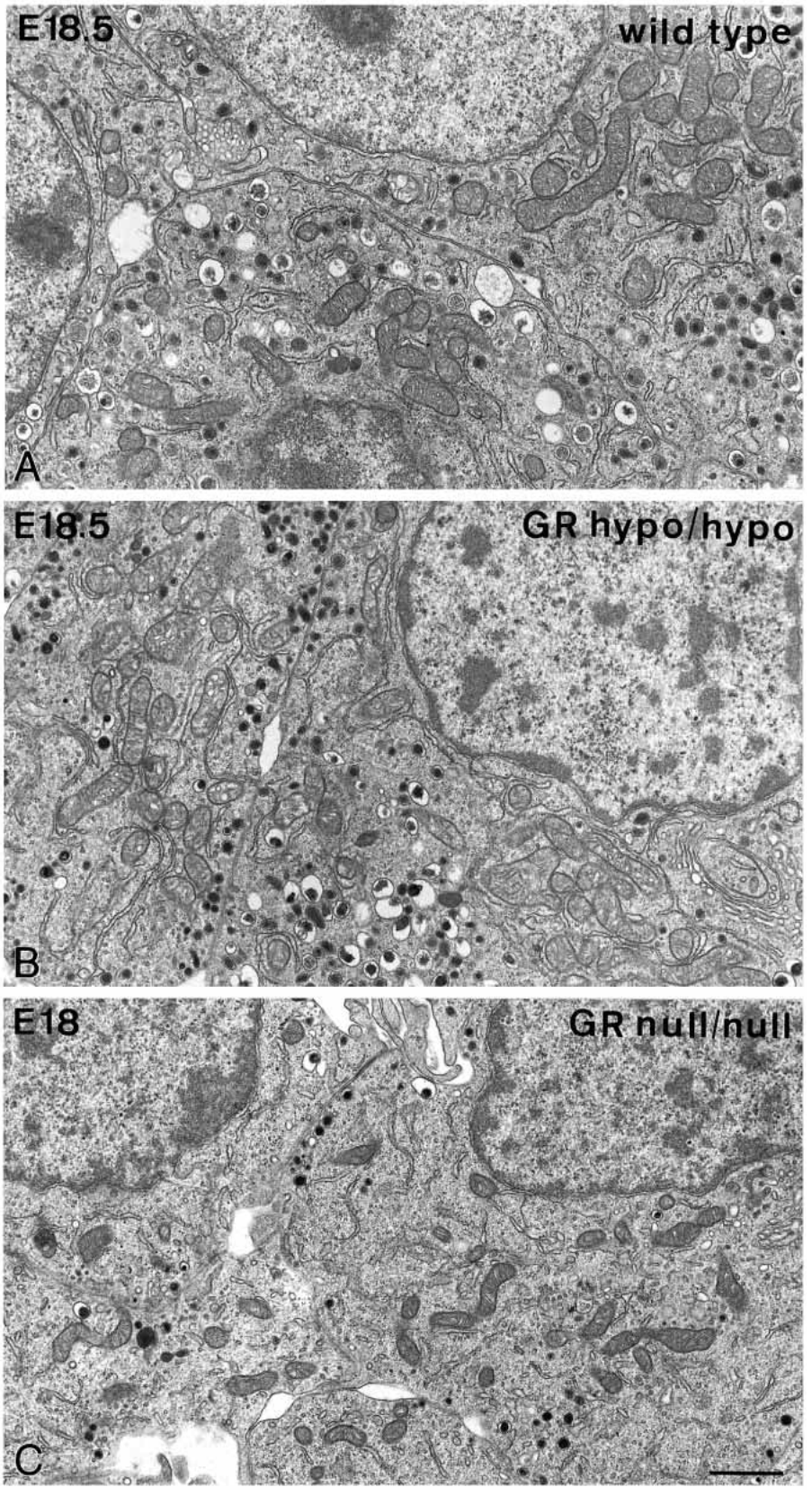

Fig. 6. Electron micrographs showing normal ultrastructural features of chromaffin cells at E18.5 in wild-type, $GR^{hypo/hypo}$ and $GR^{null/null}$ mice. Bar, 1 μm.

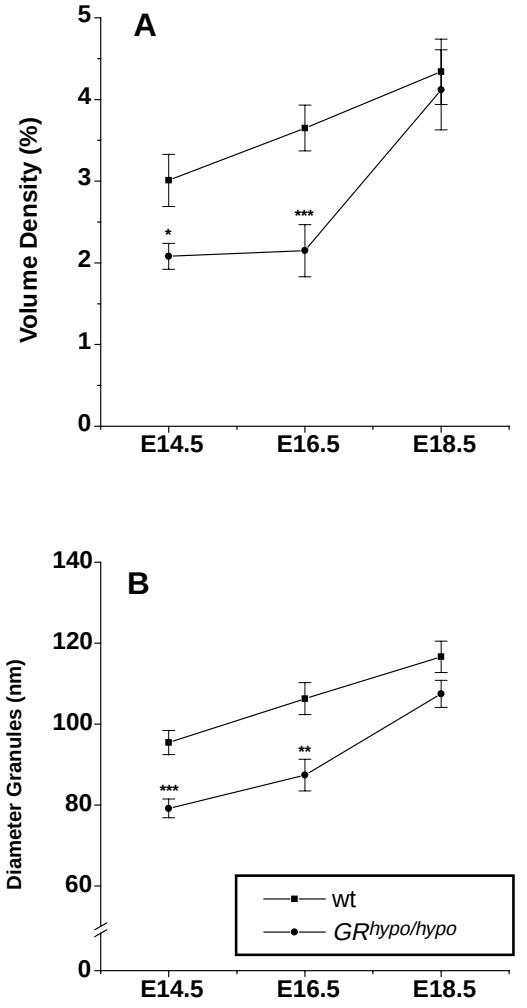

Fig. 7. Morphometric analysis of the parameters 'volume density' (i.e. percentage of cell area occupied by chromaffin granules) and 'granule diameter'. There are slight differences in these parameters between wild-type and $GR^{hypo/hypo}$ mice at E14.5 and E16.5. However, at E18.5 both parameters are not significantly different in wild-type and *GR*-deficient mice. **P*<0.05, ***P*<0.01, ****P*<0.001.

catecholaminergic cells (Tiveron et al., 1996) are not significantly different from control littermates at any ontogenetic time point studied. We assume that the expansion of the adrenal cortex (Cole et al., 1995) and massive increase in proliferative activity of cortical cells in $GR^{hypo/hypo}$ and $GR^{null/null}$ mice (S. F., unpublished) may account for the disintegration of the adrenal medulla in *GR*-deficient animals. Normal numbers of chromaffin cells in *GR*-deficient mice also precludes that a putative chromaffin sublineage, as e.g. the adrenaline sublineage, had been selectively eliminated leaving exclusively noradrenaline synthesizing cells. $GR^{hypo/hypo}$ and $GR^{null/null}$ chromaffin cells display all typical ultrastructural and many chemical features, which distinguish them from sympathetic neurones. However, they lack PNMT and SgII, which are not specific markers for the chromaffin phenotype. With regard to PNMT, it should be noted that a large proportion of intra- and extraadrenal chromaffin cells in mammals do not synthesize adrenaline (Coupland, 1953, 1965, 1972; Böck, 1982), and, conversely, that a subpopulation of sympathetic principal neurones in birds expresses PNMT (Teitelman et al., 1984). Moreover, PNMT in mammalian CNS adrenergic neurons (Bohn et al., 1981) and submammalian chromaffin cells (Holzbauer and Sharman, 1972) is not regulated by glucocorticoids (Wurtman et al., 1968). The granins SgII and CgB occur in many different types of neurones and neuroendocrine cells (Huttner et al., 1991; Rosa and Gerdes, 1994), and their expression in a given type of cell varies significantly between mammalian species (Winkler and Fischer-Colbrie, 1992). Together, this suggests that all of the above markers are not per se indicative of chromaffin cell differentiation and, consequently, that their expression may be altered without loosing the chromaffin phenotype.

Previous in vitro studies with sympathoadrenal progenitor cells have suggested two sequential glucocorticoid effects on SA progenitors and developing chromaffin cells

Developing chromaffin cells and their progenitors have been extensively used in the past two decades as model systems for studying how environmental signals determine cell fate. Following the discovery that chromaffin cells cultured from neonatal rats can switch their phenotype to that of sympathetic neurones when they are treated with NGF or several other neurotrophic factors (Unsicker et al., 1978, 1984, 1985a,b; Doupe et al., 1985; Claude et al., 1988; Stemple et al., 1988), the in vitro development of SA progenitors isolated from early sympathetic ganglionic and adrenal anlagen by fluorescence-activated cell sorting using monoclonal antibodies to the HNK-1 epitope has been extensively studied. Cell culture studies by Michelsohn and Anderson (1992) had indicated that glucocorticoids were not only essential for the induction of PNMT, but also important for inhibiting neurite growth of SA progenitors isolated from E14.5 adrenal glands, i.e. for suppressing neuronal development. Both effects were specifically mediated by the glucocorticoid receptor type II (i.e. the GR). Progesterone elicited effects similar to those of glucocorticoids, but these effects were clearly mediated by the GR. Moreover, specifically blocking the mineralocorticoid receptor did not interfere with glucocorticoid-induced neurite inhibition or induction of PNMT. From these in vitro data we would have predicted that in *GR*-deficient mice SA progenitors are not channelled into the chromaffin pathway of differentiation and either maintain their progenitor features, die or acquire neuronal phenotypes. None of these fate applies, as shown by our results.

Chromaffin cells of *GR*-deficient mice do not display neuronal traits

The present data of $GR^{hypo/hypo}$ and $GR^{null/null}$ SA progenitors and adrenal medullary cells are fully consistent with the notion that these cell lack key neuronal markers that would be indicative of their conversion into sympathetic neurones. Ultrastructurally, they do not at all resemble sympathetic neurones. Sympathetic neurones display only very few large dense core vesicles (LDV; vesicle diameter: 80-120 nm, cf. Grillo, 1966 and Eränkö, L., 1972) within their cell bodies, whereas chromaffin cells have numerous and larger (100-160 nm) LDV (=chromaffin granules), consistent with our measurements (cf. Coupland, 1972; Coupland and Tomlinson, 1989). The average size of the LDV core in wild-type and *GR*-deficient chromaffin cells did not significantly differ at E18.5

consistent with the notion that chromaffin LDV in *GR*-deficient animals were no less mature than in wild types. Moreover, adrenal chromaffin cells in $GR^{hypo/hypo}$ and $GR^{null/null}$ mice lacked the intermediate filament peripherin from E13.5 through E18.5. This is consistent with the notion that chromaffin cells have not shifted towards a neuronal phenotype. It also precludes that chromaffin cells of *GR*-deficient mice might have adopted a neuronal phenotype early in their development and might have acquired neuroendocrine features at later time points in development. Moreover, chromaffin cells from wild-type and $GR^{null/null}$ mice extend neurites in vitro in similar proportions, spontaneously or when being stimulated with NGF, further strengthening the view that loss of glucocorticoid signalling has not promoted neuronal traits by shifting the responsiveness towards NGF. Taken together, these data provide compelling evidence that SA progenitors of $GR^{hypo/hypo}$ and $GR^{null/null}$ mice have failed to adopt traits of sympathetic neurones.

Possible implications of cell culture conditions for generating a concept of glucocorticoid requirement for chromaffin cell development

The concept of an essential role of glucocorticoids in preventing neuronal differentiation in SA progenitors and channelling them into the chromaffin lineage has largely been built on in vitro evidence. There is no doubt that glucocorticoids effectively block neuronal development and neurite formation of SA progenitors, embryonic and early postnatal chromaffin cells in culture. In particular, neurite formation induced by NGF in early postnatal chromaffin cells that do not form neurites spontaneously can be fully prevented in the presence of glucocorticoid (Unsicker et al., 1978). However, these in vitro studies that have helped to conceptualize the 'glucocorticoid requirement' in chromaffin cell development also raise several concerns. 60% of cultured SA progenitors isolated from E14.5 adrenals form processes after 24 hours on a collagen/laminin substratum in the presence of 10% serum (Michelsohn and Anderson, 1992). Both this substratum and the sera may contain substantial amounts of growth factors that could facilitate neurite growth, including FGF. Furthermore, the possibility that cultured cells rapidly upregulated their own synthesis of neurite-promoting factors that could act in auto-/paracrine fashion has never been investigated. The fact that glucocorticoid only inhibits about 50% of the processes grown by SA progenitors whereas mammalian intra-adrenal chromaffin cell in situ never produce processes has never been satisfactorily explained. Together, the previous in vitro data and underlying experimental designs leave substantial doubts as to whether they comply with the in vivo situation and whether they may reflect a pharmacological rather than a physiological action of glucocorticoids.

A new putative scenario for the development of the SA lineage

The present analyses of the chromaffin cell phenotype in *GR*-deficient mice do not support the notion of an essential role of glucocorticoid signalling mediated through the GR in chromaffin cell development. Our data strongly suggest that the role of glucocorticoids is apparently restricted to modulating, directly or indirectly, the expression of a number of genes, as *PNMT*, *SgII* and *CgB*, none of which is unequivocally responsible for the chromaffin phenotype. However, our interpretation is based on the assumption that *GR*-deficient mice have not generated glucocorticoid-independent regulators of chromaffin cell development. Thus, one could speculate that mineralocorticoid receptors, by an unknown mechanism, might become responsive to glucocorticoids and transmit glucocorticoid-specific signals.

How, then, could the development of the distinct phenotypes of sympathetic neurones and chromaffin cells be regulated? SA progenitors migrating into the adrenal anlagen, like those forming sympathetic ganglionic anlagen, are both initially exposed to the same 'adrenergic' signals, which have been identified as members of the family of bone morphogenetic proteins (Shah et al., 1996; Reismann et al., 1996). It is conceivable, however, that subsequent signals or combinations of signals required for stabilizing the neuronal phenotype and neurite formation are spatially confined, reaching presumptive sympathetic neurons but not intraadrenal chromaffin cells. Alternatively, the adrenal environment may provide inhibitors of neuronal differentiation. Finally, chromaffin cells might be reached by neurite-repulsive cues, such as the Eph receptors and their ligands (see Gale and Yankopoulos, 1997, for a review), semaphorins (see Mark et al., 1997, for a review) or repulsive components within the extracellular matrix.

In summary, the present study deprives glucocorticoids of their key role in determining the fate of SA progenitors and opens avenues for a search for alternative molecules that may be implicated in chromaffin cell development.

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REFERENCES

Anderson, D. J. (1993). Molecular control of cell fate in the neural crest: The sympathoadrenal lineage. *Annu. Rev. Neurosci*.**16,** 129-158.

Anderson, D. J. and Axel, R. (1986). A bipotential neuroendocrine precursor whose choice of cell fate is determined by NGF and glucocorticoids. *Cell* **47,** 1079-1090.

Anderson, D. J. and Michelsohn, A. (1989). Role of glucocorticoids in the chromaffin-neuron developmental decision. *Int. J. Dev. Neurosci*.**12,** 83-94.

Bohn, M. C., Goldstein, M. and Black, I. (1981). Role of glucocorticoids in expression of the adrenergic phenotype in rat embryonic adrenal gland. *Dev. Biol.* **82,** 1-10.

Böck, P. (1982). The paraganglia. In *Handbuch der mikroskopischen Anatomie des Menschen* (ed. A. Oksche and L. Vollrath) VI/8, Berlin, Heidelberg: Springer-Verlag.

Bravo, R., Frank, R., Blundall, P. A. and MacDonald-Bravo, H. (1987). Cyclin/PCNA is the auxiliary protein of DNA polymerase delta. *Nature* **326,** 515-517.

Claude, P., Parada, I. M., Gordon, K. A., D'Amore, P. A. and Wagner, J. A. (1988). Acidic fibroblast growth factor stimulates adrenal chromaffin cells to proliferate and to extend neurites, but is not a long term survival factor. *Neuron* **1,** 783-790.

Cole, T. J., Blendy, J. A., Monaghan, A. P., Krieglstein, K., Schmid, W., Aguzzi, A., Fantuzzi, G., Hummler, E., Unsicker, K. and Schütz, G. (1995). Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. *Genes Dev.* **9,** 1608-1621.

Coupland, R. E. (1953). On the morphology and adrenaline-noradrenaline content of chromaffin tissue. *J. Endocr*. **9,** 194-203.

Coupland, R. E. (1965). *The Natural History of the Chromaffin Cell*. London:.Longmans, Green and Co, Ltd.

Coupland, R. E. (1972). The chromaffin system. In *Catecholamines* Handbook of Experimental Pharmacology. Vol 33 (ed. H. Blaschko and E. Muscholl), pp 16-45. Berlin, Heidelberg, New York: Springer Verlag.

Coupland, R. E. and Tomlinson, A. (1989). The development and maturation

of adrenal medullary chromaffin cells of the rat in vivo: a descriptive and quantitative study. *Int. J. Dev. Neurosci*. **7,** 419-438.

Díaz-Miranda, L., Pardo-Reoyo, C. and García-Arrarás, J. E. (1996). Galanin-like immunoreactivity in the echinoderm *Holothuria glaberrima. Cell Tissue Res.* **286**, 385-391.

Doupe, A. J., Landis, S. C. and Patterson, P. H. (1985). Environmental influences in the development of neural crest derivatives: glucocorticoids, growth factors and chromaffin cell plasticity. *J. Neurosci.* **5,** 2119-2142.

Eränkö, L. (1972). Ultrastructure of the developing sympathetic nerve cell and the storage of catecholamines. *Brain Res*. **46,** 159-175.

Eränkö, O. (1972). Light and electron microscopic histochemical evidence of granular and non granular storage of catecholamines in the sympathetic ganglion of the rat. *Histochem. J*. **4,** 213-224.

Finotto, S., Mekori Y. and Metcalfe, D. D. (1997). Glucocorticoids decrease tissue mast cell number by reducing the production of the c-kit ligand, Stem Cell Factor, by resident cells. *J. Clin. Invest*. **99,** 1721-1728.

Gale, N. W. and Yankopoulos, G. D. (1997). Ephrins and their receptors: a repulsive topic? *Cell Tiss. Res*. **290**, 227-242.

García-Arrarás, J. E., Chanconie, M. and Fontaine-Perús, J. (1984). *In vivo* and *in vitro* development of somatostatin-like immunoreactivity in the peripheral nervous system of quail embryos. *J. Neurosci.* **4**, 1549-1558.

García-Arrarás, J. E., Lugo-Chinchilla, A. M. and Chévere-Colón, I. (1992). The expression of neuropeptide Y immunoreactivity in the avian sympathoadrenal system conforms with two models of coexpression development for neurons and chromaffin cells. *Development* **115**, 617-627.

Grant, N. J., Lion, C., Aunis, D. and Langley, K. (1992). Cellular localization of the neuronal cell adhesion molecule L1 in adult rat neuroendocrine and endocrine tissues:comparisons with NCAM. *J. Comp. Neurol.* **35,** 548-558.

Grant, N. J., König F., Aunis, F. and Langley, K. (1994). Expression of GAP-43 (neuromodulin) during the development of the rat adrenal gland. *Dev. Brain Res.* **82,** 265-276.

Grillo, M. A. (1966). Electron microscopy of sympathetic tissue. *Pharmacol. Rev***. 18,** 387-399.

Groves, A. K., George, K. M., Tissier-Seta, J. P., Engel, J. D., Brunet, J. F. and Anderson, D. J. (1995). Differential regulation of transcription factor gene expression and phenotypic markers in developing sympathetic neurons. *Development* **121,** 887-901.

Gu, H., Marth, J. D., Orban, P. C., Mossmann, H. and Rajewsky, K. (1994). Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science* **265**, 103-106.

Holzbauer, M. and Sharman, D. F. (1972). The distribution of catecholamines in vertebrates. In *Catecholamines* Handbook of Experimental Pharmacology, Vol. 33 (ed. H. Blaschko and E. Muscholl), pp. 110-185. Berlin-Heidelberg-New York: Spinger-Verlag.

Huttner, W. B., Gerdes, H. H. and Rosa, P. (1991). The granin family. *Trends Biochem. Sci.* **1,** 1-19.

Kühn, R., Rajewsky, K. and Müller, W. (1991). Generation and analysis of interleukin-4 deficient mice. *Science* **254,** 707-710.

Landis, S. C. and Patterson, P. H. (1981). Neural crest cell lineages. *Trends Neurol. Sci.* **4,** 172-175.

Leon, C., Grant, N. J., Aunis, D. and Langley, K. (1992). Cellular localization of the neuronal cell adhesion molecule L1 in adult rat neuroendocrine and endocrine tissues:comparisons with NCAM. *J. Comp. Neurol.* **325,** 548-558.

Lewis, E. J., Harrington, C. A. and Chikaraishi, D. M. (1987). Transcriptional regulation of the tyrosine hydroxylase gene by glucocorticoid and cyclic AMP. *Proc. Natl. Acad. Sci. USA*. **8,** 3550-3554.

Mark, M. D., Lohrum, M. and Püschel, A. W. (1997). Patterning neuronal connections by chemorepulsion: the semaphorins. *Cell Tiss. Res.* **290**, 299-306.

Michelsohn, A. M. and Anderson, D. J. (1992). Changes in competence determine the timing of two sequential glucocorticoid effects on sympathoadrenal progenitors. *Neuron* **8,** 589-604.

Peter, D., Jimenez, J., Liu, Y., Kim, J. and Edwards, R. H. (1994). The chromaffin granule and synaptic vesicle amine transporters differ in substrate recognition and sensitivity to inhibitors. *J. Biol. Chem.* **269,** 7231-7237.

Peter, D., Liu, Y., Sternini, C., De Giorgio, R., Brecha, N. and Edwards, R. H. (1995). Differential expression of two vesicular monoamine transporters. *J. Neurosci*. **15**, 6179-6188.

Reissmann, E., Ernsberger, U., Francis-West, P. H., Rueger, D., Brickell, P. M. and Rohrer, H. (1996). Involvment of bone morphogenetic protein-4 and bone morphogenetic protein-7 in the differentiation of the adrenergic phenotype in developing sympathetic neurons. *Development* **122,** 2079-2088.

Rosa, P. and Gerdes, H. H. (1994). The granin protein family:Markers for neuroendocrine cells and tools for the diagnosis of neuroendocrine tumors. *J. Endocrinol. Invest*. **17,** 207-225.

Rosa, P., Hille, A., Lee, R. W. H., Zanini, A., DeCamilli, P. and Huttner, W. B. (1985). Secretogranins I and II: two tyrosine-sulfated secretory proteins common to variety of cells secreting peptides by the regulated pathway. *J. Cell Biol*. **10,** 1999-2011.

Schober, A., Minichiello, L., Keller, M., Huber, K., Layer, P. G., Roig-Lopez, J. L., Garcia-Arraras, J. E., Klein, R. and Unsicker, K. (1997). Reduced acetylcholinesterase (AChE) activity in adrenal medulla and loss of sympathetic preganglionic neurons in TrkA-deficient, but not TrkB-deficient, mice. *J. Neurosci*. **17**, 891-903.

Schober, A., Wolf, N., Huber, K., Hertel, R., Krieglstein, K., Minichiello, L., Kahane, N., Widenfalk, J., Kalcheim, C., Olson, L., Klein, R., Lewin, G. R. and Unsicker, K. (1998). TrkB and neurotrophin-4 are important for development and maintenance of sympathetic preganglionic neurons innervating the adrenal medulla. *J. Neurosci*. **18**, 7272-7284.

Seidl, K. and Unsicker, K. (1989a). The determination of the adrenal medullary cell fate during embryogenesis. *Dev. Biol.* **136,** 481-490.

Seidl, K. and Unsicker, K. (1989b). Survival and neuritic growth of sympathoadrenal (chromaffin) precursor cells in vitro. *Int. J. Dev. Neurosci*. **7,** 465-473.

Shah, N. M., Groves, A. K. and Anderson, D. J. (1996). Alternative neural crest cell fates are instructively promoted by TGF beta superfamily members. *Cell* **85,** 331-343.

Stemple, D. L., Mahanthappa, N. K. and Anderson, D. J. (1988). Basic FGF induces neuronal differentiation, cell division, and NGF dependence in chromaffin cells: a sequence of events in sympathetic development. *Neuron* **1,** 517-525.

Teitelman, G., Skaper S., Baker,H., Park, D. H., Joh, T. H. and Adler, R. (1984). Expression of phenylethanolamine N-methyltransferase in sympathetic neurons and extraadrenal chromaffin tissue of chick embryos in vivo and in vitro. *Dev. Brain. Res.* **13,** 283-291.

Tischler, A. S., Ruzicka, L. A., Donahue, S. R. and DeLellis, R. A. (1989). Chromaffin cell proliferation in the adult rat adrenal medulla. *Int. J. Dev. Neurosci.* **7,** 439-448.

Tischler, A. S., McClain, R. M., Childers, H. and Downing, J. (1991). Neurogenic signals regulate chromaffin cell proliferation and mediate the mitogenic effect of reserpine in the adult rat adrenal medulla. *Lab. Invest.* **65,** 374-376.

Tiveron, M. C., Hirsch M. R. and Brunet, J. F. (1996). The expression pattern of the transcription factor Phox-2 delineates synaptic pathways of the autonomic nervous system. *J. Neurosci.* **16,** 7649-7660.

Tronche, F., Kellendonk, C., Reichardt, H. M. and Schütz, G. (1998). Genetic dissection of glucocorticoid receptor function in mice. *Curr. Opin. Gen. Dev.* **8**, 532-538-

Unsicker, K. (1993). The chromaffin cell: paradigm in cell, developmental, and growth factor biology. *J. Anat.* **183**, 207-221.

Unsicker, K. and Chamley, J. H. (1977). Growth characteristics of postnatal rat adrenal medulla in culture. *Cell Tissue Res.* **177,** 247-268.

Unsicker, K., Krisch, B., Otten, J. and Thoenen, H. (1978). Nerve growth factor-induced fiber outgrowth from isolated rat adrenal chromaffin cells: Impairment by glucocorticoids. *Proc. Natl. Acad. Sci. USA* **75,** 3498-3502.

Unsicker, K., Vey, J., Hofman, H. D., Müller, T. H. and Wilson, A. J. (1984)**.** Glioma cell-conditioned medium induces neurite outgrowth and survival of rat chromaffin cells in vitro: Comparison with the effects of nerve growth factor. *Proc. Natl. Acad. Sci. USA*. **81,** 2242-2246.

Unsicker, K., Skaper, S. D. and Varon, S. (1985a). Neurotrophic and neurite promoting factors: Effects on early postnatal chromaffin cells from rat adrenal medulla. *Dev. Brain Res.* **17,** 117-129.

Unsicker, K., Skaper, S. D. and Varon, S. (1985b). Developmental changes in the responses of rat chromaffin cells to neuronotrophic and neurite promoting factors. *Dev. Biol*. **111,** 425-433.

Unsicker, K., Seidl, K. and Hofman, H.-D. (1989). The neuro-endocrine ambiguity of sympathoadrenal cells. *Int. J. Devl. Neurosci.* **7**, 413-417.

Verhofstad, A. A. J. (1993). Kinetics of adrenal medullary cells. *J. Anat.* **183,** 315-326.

Waseem, N. H. and Lane, D. P. (1990). Monoclonal antibody analysis of the proliferating cell nuclear antigen (PCNA). Structural conservation and the detection of a nucleolar form. *J. Cell Sci.* **69,** 121-129.

Weihe, E., Schäfer, M. K. H., Erichson, J. D. and Eiden, L. E. (1994). Localization of vescicular monoamine transporter isoforms (VMAT1 and VMAT2) to endocrine cells and neurons in rat. *J. Mol. Neurosci*. **5,** 149-164.

Winkler, H. (1993). The adrenal chromaffin granule: a model for large dense core vesicles of endocrine and nervous tissue. *J. Anat.* **183,** 237-252.

Winkler, H. and Fischer-Colbrie, R. (1992). The chromogranins A and B: the first 25 years and future perspectives. *Neuroscience* **49**, 497-528.

Wurtman, R. J. and Axelrod, J. (1965). Adrenaline synthesis: Control by the pituitary gland and adrenal glucocorticoids. *Science* **150,** 237-252.

Wurtman R. J., Axelrod, J., Vesell, E. S. and Ross, G. T. (1968). Species differences in inducibility of phenylethanolamine-N-methyltransferase. *Endocrinology* **82,** 584-590.