Strain-specific steroidal control of pituitary function

Sang-Nam Lee, Bonnie Peng¹, Roxane Desjardins², John E Pintar¹, Robert Day² and Iris Lindberg

Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, New Orleans, 1901 Perdido Street, New Orleans, Louisiana 70112, USA

¹Department of Neuroscience and Cell Biology, University of Medicine and Dentistry of NJ, Piscataway, New Jersey 08854, USA

²Département de Pharmacologie, Faculté de Médecine et Institut de Pharmacologie de Sherbrooke, Université de Sherbrooke, Québec, Canada J1H 5N4

(Requests for offprints should be addressed to I Lindberg; Email: ilindb@lsuhsc.edu)

Abstract

We have previously shown that 7B2 null mice on the 129/SvEvTac (129) genetic background die at 5 weeks of age with hypercorticotesteronemia due to a Cushing's-like disease unless they are rescued by adrenalectomy; however, 7B2 nulls on the C57BL/6NTac (B6) background remain healthy, with normal steroid levels. Since background exerts such a profound influence on the phenotype of this mutation, we have evaluated whether these two different mouse strains respond differently to high circulating steroids by chronically treating wild-type 129 and B6 mice with the synthetic steroid dexamethasone (Dex). Dex treatment decreased the dopamine content of the neurointermediate lobes (NIL) of 129 mice, leading to NIL enlargement and increased total D₂R mRNA in the 129, but not the B6, NIL. Despite the decrease in this inhibitory transmitter, Dex-treated 129 mice exhibited reduced circulating α -melanocyte-stimulating hormone

(α -MSH) along with reduced POMC-derived peptides compared with controls, possibly due to reduced POMC content in the NIL. In contrast, Dex-treated B6 mice showed lowered cellular ACTH, unchanged α -MSH and β -endorphin, and increased circulating α -MSH, most likely due to increased cleavage of NIL ACTH by increased PC2. Dex-treated 129 mice exhibited hyperinsulinemia and lowered blood glucose, whereas Dex-treated B6 mice showed slightly increased glucose levels despite their considerably increased insulin levels. Taken together, our results suggest that the endocrinological response of 129 mice to chronic Dex treatment is very different from that of B6 mice. These strain-dependent differences in steroid sensitivity must be taken into account when comparing different lines of transgenic or knockout mice.

Journal of Endocrinology (2007) **192**, 515–525

Introduction

Prohormone convertase 1 (PC1/3) along with prohormone convertase 2 (PC2), members of the family of calcium-dependent subtilisin-like endoproteases, is predominantly expressed in neuronal and endocrine cells (Seidah *et al.* 1990, Day *et al.* 1992). Both PC1/3 and PC2 generate bioactive peptides from prohormones such as proopiomelanocortin (POMC; Benjannet *et al.* 1991), proglucagon (Rouille *et al.* 1997), and proinsulin (Bennett *et al.* 1992) by endoproteolytic processing at paired basic residues (reviewed by Seidah *et al.* 1994). PC2 and PC1/3 are both synthesized as proenzymes; the propeptide must be cleaved to generate an enzymatically active mature species (Muller & Lindberg 1999). Unlike PC1/3, the PC2 precursor requires a neuroendocrine-binding partner, 7B2, in order to generate an active enzyme molecule (Muller *et al.* 1997, Muller & Lindberg 1999). While the molecular mechanism of the PC2 and 7B2 interaction remains unclear, three subdomains within proPC2 and a 36-residue peptide within 7B2 are known to participate (Muller *et al.* 1999), and our present working hypothesis is that 7B2 blocks proPC2 from

assuming a non-activatable conformer (S-N Lee & I Lindberg, unpublished data).

The POMC precursor is expressed in both the corticotrophs of the anterior lobe (AL) and the melanotrophs of the pituitary neurointermediate lobe (NIL). However, corticotrophs express mainly PC1 mRNA with low levels of PC2 mRNA, while melanotrophs express high levels of PC2 mRNA with appreciable PC1 mRNA levels (Seidah *et al.* 1990, Day *et al.* 1992). Since the specificity of PC1/3 and PC2 is not identical, processing of the POMC precursor differs greatly between corticotrophs and melanotrophs (Mains & Eipper 2000). In AL corticotrophs, POMC is processed mainly into adreno-corticotrophic hormone (ACTH) and β -lipotropin as well as into lesser amounts of β -endorphin, while in NIL melanotrophs, POMC-derived peptides are cleaved to a variety of smaller bioactive peptides, such as α -melanocyte-stimulating hormone (α -MSH) and β -endorphin (Mains & Eipper 2000). Secretion of POMC-derived peptides in both cell types is stimulated by both the hypothalamic peptides, arginine vasopressin and corticotropin-releasing factor (CRF; Vale *et al.* 1981, Lundblad & Roberts 1988). Control of

expression and secretion of POMC-derived peptides is negatively regulated in a cell type-dependent manner; in the AL, glucocorticoids exert a negative feedback effect by a direct action on corticotrophs (Raymond *et al.* 1979) and by inhibiting hypothalamic CRF (Suda *et al.* 1984). By contrast, in the NIL, POMC expression and secretion are primarily under direct tonic inhibitory control by A14 periventricular-hypophysial dopaminergic neurons (Goudreau *et al.* 1995). *In vivo* chronic administration with D₂R agonists decreases melanotroph POMC biosynthesis, the secretion of α -MSH and β -endorphin (Chen *et al.* 1983, Beaulieu *et al.* 1984), and the melanotroph proliferation rate (Chronwall *et al.* 1987). In addition, removal of dopaminergic axons within the rat intermediate lobe by the administration of 6-hydroxydopamine increases melanotroph proliferation (Rychter & Stepien 1977, Gary & Chronwall 1992). Thus, POMC expression is controlled differently in the two lobes of the pituitary.

Previous data obtained using the PC2 and 7B2 null mouse models have shown that both nulls show defective prohormone processing *in vivo* (Furuta *et al.* 1998, Westphal *et al.* 1999, Laurent *et al.* 2002). Interestingly, PC2 and 7B2 null mice exhibit exceedingly different phenotypes; PC2 null mice are healthy except for slight hypoglycemia and runting (Furuta *et al.* 1997), while 7B2 null mice die at 5 weeks due to a Cushing's disease-like disorder (Westphal *et al.* 1999). Cushing's disease normally arises from adrenal hypercorticotesteronism as a result of ACTH hypersecretion from anterior pituitary (reviewed by Shomali & Hussain 2000); the 7B2 null instead develops a Cushing's-like disease via greatly enhanced secretion of ACTH from the NIL (Westphal *et al.* 1999). Hypercorticotesteronism is directly involved in the lethal phenotype, as adrenalectomy rescues the 7B2 129 null from death (Laurent *et al.* 2002).

However, the PC2 and 7B2 nulls were not generated on identical backgrounds; the PC2 nulls are in a mixed 129×B6 background, while the 7B2 nulls are in a pure 129 genetic background. The profound effect of background on the 7B2 mutation was clearly demonstrated by transfer of the 7B2 null onto a pure B6 background, where it does not exhibit signs of disease (Peinado *et al.* 2005). Conversely, the movement of the PC2 null to a 129 background results in the development of illness similar to the 7B2 null after six generations of backbreeding (Peinado *et al.* 2005). Since ACTH hypersecretion and hypercorticotesteronism have been directly implicated in the development of illness, we speculated that the two strains are differentially sensitive to ACTH and steroids. Indeed, we have confirmed that 129 mice possess a more sensitive adrenocortical response to ACTH than B6 mice using ACTH challenge experiments (Peinado *et al.* 2005).

In order to test for other strain-dependent effects of excess circulating steroids, in this study we have treated wild-type 129 and B6 mice with chronic dexamethasone (Dex) to reproduce the effect of excess circulating steroid in a manner similar to that observed in 7B2 nulls. Dex is a potent synthetic corticosteroid that mimics the action of endogenous steroids

in the suppression of AL ACTH synthesis and secretion (Murphy 1991). We have examined the effect of Dex on the hypothalamo-pituitary-adrenal (HPA) axis in both 129 and B6 wild-type mice and determined cellular and/or circulating plasma levels of ACTH, α -MSH, β -endorphin, dopamine, and insulin using RIA, and blood glucose levels. Lastly, we have performed ultrastructural analyses of melanotrophs and *in situ* hybridization of D₂R expression levels in NIL. Our results, described below, reveal considerable strain differences in the response to steroids in most of the parameters measured.

Materials and Methods

Animals

Four-week-old 129/SvEvTac and C57BL/6NTac wild type (WT) males were commercially purchased from Taconic Farms (Germantown, NY, USA). Mice were individually housed in an Association for Assessment and Accreditation of Laboratory Animal Care-approved animal care facility. Killing procedures were approved by the Louisiana State University Health Sciences Center (LSUHSC) animal care committee.

Dexamethasone administration

Four-week-old mice were given 10 µg/ml Dex (Sigma) in the drinking water for a week before they are killed; this timing was chosen to approximate the steroid susceptibility of the 129 7B2 null in the week prior to death. Pituitaries were removed and dissected into ALs and NILs. Separate lobes were individually homogenized via sonication either in 60 µl 0.5 M HClO₄ containing 10 mg/ml sodium bisulfite for dopamine assay or in 250 µl ice-cold 5 M acetic acid with 2 mg/ml BSA for ACTH and α -MSH assays. The samples were centrifuged for 15 min at 17 383 g at 4 °C and then clear supernatants were individually harvested into fresh tubes. Serum was prepared from trunk blood obtained from mice killed by decapitation at the same time of the day (1030–1230 h). Clotted blood was centrifuged briefly to separate the serum from cells and then collected individually in fresh tubes. All samples were stored frozen at –70 °C until use.

ACTH assays

Ten to twenty microliters of a 1/200 dilution of pituitary samples (ALs or NILs) or 50–100 µl sera prepared from either 129 control mice, Dex-treated 129 mice, B6 mice, or Dex-treated B6 mice were assayed in duplicate using the two-site Nichols human ACTH_{1–39} assay kit (Nichols Institute, San Juan Capistrano, CA, USA). The ¹²⁵I-ACTH antibody used in this kit is directed to both N-terminal and C-terminal regions of intact ACTH molecule and does not recognize ACTH cleavage products. Dilutions were performed in RIA buffer (100 mM sodium phosphate, pH 7.4, containing 0.1% heat-treated BSA, 50 mM NaCl, and 0.1% sodium azide).

Radioactivity was determined using a Wallac 1470 Wizard gamma counter (Perkin-Elmer Life and Analytical Sciences, Shelton, CT, USA).

Corticosterone assays

Five to ten microliters of sera were assayed using the Immuchem Double Antibody corticosterone ^{125}I RIA kit (M P Biomedicals, Orangeburg, NY, USA).

α -MSH and β -endorphin assays

Twenty microliters (for ALs) or 10 μl (for NILs) of a 1/200 dilution pituitary samples were subjected to assay in duplicate. The polyclonal anti- α -MSH antiserum was commercially purchased from Chemicon (Temecula, CA, USA) and the polyclonal antiserum Bunny 3 (provided by Dr Robert Dore, University of Denver, Denver, CO, USA) was used to detect β -endorphin. ^{125}I -Labeled α -MSH and β -endorphin were prepared by the chloramine-T method, originally described by Hunter & Greenwood (1962). RIAs were carried out according to protocols described previously (Fortenberry *et al.* 1999). Samples were incubated with 10 000 c.p.m. iodinated peptide and the appropriate dilution of rabbit antiserum in a final volume of 300 μl at 4 °C overnight. To separate the antibody-bound labeled peptide from the unbound labeled peptide, 1 ml 25% polyethylene glycol and 100 μl 7.5% carrier bovine γ -globulin (in PBS) were added. The samples were vortexed, kept on ice for 30 min, and then centrifuged for 20 min at 3000 *g* at 4 °C using a Sorvall RT6000B refrigerated centrifuge. The supernatant was aspirated and the radioactivity in the pellets was determined. For serum samples, 100 μl sera were assayed in duplicate using the EURIA- α -MSH RIA kit (Alpco Diagnostics, Windham, NH, USA). This anti- α -MSH-antiserum is directed toward the C-terminal region of the α -MSH molecule and does not recognize ACTH.

Dopamine assays

Ten microliters of AL or NIL samples were assayed in duplicate using a dopamine RIA kit (Rocky Mountain Diagnostics, Colorado Springs, CO, USA).

Glucose assays

For glucose analyses, trunk blood collected from decapitated mice was analyzed immediately after killing, using a Freestyle blood glucose monitoring system (Therasense, Alameda, CA, USA).

Insulin and glucagon assays

Twenty-five microliters of sera (insulin assay) or 50–100 μl sera (glucagon assay) were assayed using the Linco RIA kit (St Charles, MI, USA).

Western blotting

All antisera were raised in rabbits against peptides conjugated to keyhole limpet hemocyanin. The antiserum against PC2 (LS18) was directed against the COOH-terminus of mature PC2 (Shen *et al.* 1993). The antiserum against 7B2 (LS13) was raised against residues 23–39 of 7B2 (Zhu & Lindberg 1995). The antiserum against PC1 (LS2) was directed against the amino terminus of mature PC1 (Vindrola & Lindberg 1992, Zhu & Lindberg 1995). The antiserum against POMC (LS41) was directed against ACTH(1–24) (Fortenberry *et al.* 2002). Thirty microliters of pituitary (ALs or NILs) samples solubilized directly in Laemmli sample buffer were applied to the gels. Samples were subjected to electrophoresis on 10% Criterion Tris-HCl (Bio-Rad) SDS gels, followed by western blotting using the respective antisera. Proteins were transferred from gels to nitrocellulose membranes, and the membranes were preincubated in 5% non-fat milk in TBS for 30 min at room temperature prior to incubation overnight at 4 °C with antiserum 2B6 diluted 1:1000 in milk. Membranes were washed thrice with tris buffer saline (TBS) containing 0.05% Tween followed by incubation at room temperature for 1.5 h with secondary antibody (goat anti-rabbit IgG coupled to alkaline phosphatase) diluted 1:10 000 in milk. Membranes were then washed once with TBS containing 0.05% Tween and twice with TBS alone, and then developed with 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt/*p*-nitro blue tetrazolium chloride. For western blotting using horseradish peroxidase (HRP) conjugate as secondary antibody, membranes were incubated with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) for 1 min, and the chemiluminescent bands were revealed using a Fluor-S Max MultiImager System.

In situ hybridization

Pituitaries were removed, embedded in OCT compound (Miles, Elkhart, IN, USA), frozen in 2-methylbutane on dry ice powder, and stored at –20 °C before sectioning. Sections (10 μm) were mounted onto Superfrost/Plus slides (Fisher Scientific, Fairlawn, NJ, USA) and stored at 70 °C until use.

The specific D₂R complementary RNA and sense-strand control probes were transcribed using ^{35}S -labeled UTP (New England Nuclear, Boston, MA, USA), Riboprobe System II transcription buffers (Promega), either T3 or T7 RNA polymerase (Stratagene, La Jolla, CA, USA), and a linearized plasmid construct that contains a 295 bp cDNA fragment corresponding to the D₂R exon 2 coding region.

In situ hybridization was performed as described by Snyder *et al.* (1998). Autoradiography was performed at 4 °C using a 1:1 dilution of Kodak NTB2 emulsion (Eastman Kodak) and HPLC grade dH₂O for 1–4 weeks. Eight slices were taken per pituitary and the density given represents 12 images from six sections. Densities (grains/area) were determined using NIH 1.62 image software. In all cases, hybridization with negative controls

(sense-strand cRNA probes) in adjacent sections yielded only low background.

Electron microscopy

Sample preparation for electron microscopy was carried out using methods described previously (Day *et al.* 1987). Pituitaries from animals were removed, fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4), embedded in Epon, and sectioned. The sectioned samples were stained with 1% uranyl acetate and 6% lead citrate (pH 10). Pictures of identical surface areas were taken at a magnification of $\times 7000$.

Statistical analysis

Data were analyzed using Student's *t*-test. Results are expressed as the mean $\pm$ S.E.M. of the number of samples indicated.

Results

We treated 5-week-old 129 and B6 mice with the synthetic glucocorticoid Dex for 1 week, mimicking the effect of high circulating corticosteroid levels found in 7B2 nulls just prior to their death. Following dissection of the AL and NIL from whole pituitary, the levels of various pituitary and circulating hormones were determined. It was important to analyze the two pituitary lobes separately, given the fact that the release and content of POMC-derived peptides differs significantly between the two lobes. Most of the Dex-treated 129 animals showed symptoms of illness such as slower growth, fragile, thin skin, and poor hair coat condition. Dex-treated B6 mice showed similar, but much less severe signs of illness. Both 129 and B6 mice treated with Dex exhibited growth retardation compared with controls, as indicated by reduced body weights (Fig. 1). Although the volumes consumed by Dex-treated 129 mice were slightly more than other groups of mice, this effect did not reach statistical significance (Fig. 1).

Dex-treated 129 mice exhibit decreased circulating α -MSH levels, whereas Dex-treated B6 mice have increased circulating α -MSH levels; basal hormone levels vary between the two strains

To assess a potential differential HPA axis response to chronically administered Dex, we assayed sera from Dex-treated 129 and B6 animals for ACTH, corticosterone, and α -MSH using radioimmunoassays.

The 129 control mice exhibited significantly (twofold) higher basal circulating levels of ACTH and fourfold higher circulating corticosterone than B6 mice (Table 1). Dex treatment decreased serum ACTH and corticosterone in both strains to undetectable levels (Table 1), a normal response of the HPA axis to glucocorticoid negative feedback inhibition (Suda *et al.* 1984).

Figure 1 Dex-treated 129 and B6 mice show growth retardation (A) and no significant difference in water volumes drunk (B) ($P < 0.0584$; $n = 5$). The 129 and B6 mice were given Dex in the drinking water for a week before killing. Ctr, control.

Circulating α -MSH levels were decreased approximately 2.5-fold in Dex-treated 129 mice when compared with their controls (Table 1). In contrast, Dex-treated B6 mice showed increased circulating α -MSH levels (twofold) compared with controls (Table 1).

Dexamethasone treatment results in decreased ACTH, α -MSH, β -endorphin, and POMC levels in 129 NIL

To test whether Dex treatment affects the pituitary contents of POMC-derived peptides, we performed RIAs of ACTH, α -MSH, and β -endorphin on pituitary lobe extracts; in addition, we performed western blotting for POMC, PC1, and PC2 in 129 and B6 NIL.

Although circulating ACTH levels in 129 WT controls were higher than those in B6 WT mice (Table 1), AL ACTH levels were lower in 129 mice than in B6 mice, potentially indicating a higher secretion rate in 129 AL (Table 2). Dex treatment resulted in considerably decreased ACTH levels in the AL of both strains via the normal response of negative glucocorticoid feedback inhibition (Table 2). Additionally, β -endorphin was detected in B6 AL (Table 2), but not in 129 AL. This result indicates that the β -endorphin content of the B6 AL is considerably higher than that of the 129 AL and confirms previous data on differential processing of POMC in different strains (Crabbe *et al.* 1981).

Table 1 Comparison of the serum levels of ACTH, corticosterone, and α -MSH in 129 and B6 mice treated with dexamethasone or vehicle (Ctr). Data represent the mean $\pm$ S.E.M. of the number of animals shown

	Unit	129		B6	
		Ctr	Dex	Ctr	Dex
ACTH	pg/ml	28.9 $\pm$ 2.5* N=14	ND	13.8 $\pm$ 2.8* N=8	ND
Corticosterone	ng/ml	71.7 $\pm$ 3.1 [†] N=24	ND	17.1 $\pm$ 2.3 [†] N=21	ND
α -MSH	pg/ml	116 $\pm$ 5.9 ^{‡,} N=12	44.0 $\pm$ 2.9 [‡] N=14	19.3 $\pm$ 2.3 ^{§,} N=15	33.5 $\pm$ 2.8 [§] N=13

ND, not detectable (below the limit of detection, 1.0 pg/ml for ACTH, 2.5 ng/ml for corticosterone). *Significant statistical differences in ACTH levels were observed between 129 Ctr and B6 Ctr mice ($P=0.0012$). [†]Significant statistical differences in corticosterone levels were observed between 129 Ctr and B6 Ctr mice ($P<0.0001$). ^{‡,§,||}Significant statistical differences in α -MSH levels were observed; [‡], between Ctr and Dex in 129 mice ($P<0.0001$); [§], between Ctr and Dex in B6 mice ($P=0.0005$); ^{||}, between 129 Ctr and B6 Ctr mice ($P<0.0001$).

The basal content of ACTH in the 129 NIL was about 3.5-fold higher than that in the B6 NIL (Table 2). Although the circulating plasma α -MSH levels in 129 mice were sixfold higher than those in B6 mice (Table 1), there were no significant differences in both cellular α -MSH and β -endorphin levels in the NIL of both strains (Table 2), supporting the idea of a higher basal peptide secretion rate from 129 NIL. As shown in Table 2, Dex treatment resulted in an unexpected alteration of POMC processing in the 129 NIL; a significant 1.4-, 2-, and 1.8-fold decrease in cellular ACTH, α -MSH, and β -endorphin levels respectively were observed. In contrast, ACTH levels in Dex-treated B6 NIL were decreased when compared with controls, and α -MSH and β -endorphin levels were unaltered by Dex (Table 2).

Interestingly, the basal PC2 content of 129 NIL was significantly higher than that of B6 NIL in untreated mice, while no differences in PC1 content were observed between strains (Fig. 2A). This result suggests that the further processing of ACTH to α -MSH may be enhanced in 129 NIL compared with B6 NIL; this finding is in agreement with the considerably higher levels of basal plasma α -MSH observed in 129 mice. In addition, the total POMC contents were significantly higher in 129

mice than in B6 mice (Fig. 2B), possibly because the surface area of 129 NIL is fourfold larger than that of B6 NIL (Fig. 3A).

In Dex-treated 129 NILs, the POMC content was decreased, and PC1 and PC2 contents were unaltered (Fig. 2). In Dex-treated 129 ALs, the contents of POMC and both enzymes were unaltered (data not shown). In contrast, Dex treatment resulted in significantly decreased POMC, but increased PC2 content in B6 NIL (Fig. 2), suggesting that in B6 mice, Dex treatment facilitates the processing of ACTH to α -MSH via an increase in PC2.

Taken together with the RIA data presented in Table 1, these data imply that the decreased plasma α -MSH levels observed in Dex-treated 129 mice might result from the decreased POMC content in the NIL rather than via decreased release of α -MSH. On the other hand, in B6 NIL, Dex may increase POMC processing by upregulating the content of the prohormone convertase PC2.

In summary, Dex treatment decreased both circulating (Table 1) and pituitary (Table 2) levels of POMC-derived peptides in 129 mice (similar to the results described in Chen *et al.* 1983), but many of these effects on POMC peptides were not recapitulated in B6 mice.

Table 2 Comparison of the pituitary levels of ACTH, α -MSH, and β -endorphin (μ g/pituitary) in 129 and 129 mice treated with dexamethasone or vehicle (Ctr). Data represent the mean $\pm$ S.E.M. of the number of animals shown

		129		B6	
		AL	NIL	AL	NIL
ACTH	Ctr	0.18 $\pm$ 0.012 ^{*,†} N=11	0.07 $\pm$ 0.004 ^{§,¶} N=11	0.28 $\pm$ 0.016 ^{†,‡} N=9	0.02 $\pm$ 0.003 ^{,¶} N=10
	Dex	0.04 $\pm$ 0.006 [*] N=12	0.05 $\pm$ 0.005 ^{§,‡} N=12	0.03 $\pm$ 0.002 [†] N=11	0.01 $\pm$ 0.002 ^{,‡} N=15
α -MSH	Ctr	ND	0.31 $\pm$ 0.023 ^b N=15	ND	0.38 $\pm$ 0.038 N=13
	Dex	ND	0.14 $\pm$ 0.016 ^{b,c} N=13	ND	0.33 $\pm$ 0.023 ^c N=11
β -endorphin	Ctr	ND	0.92 $\pm$ 0.118 ^d N=5	0.38 $\pm$ 0.088 N=5	1.11 $\pm$ 0.103 N=5
	Dex	ND	0.49 $\pm$ 0.081 ^{d,e} N=6	0.24 $\pm$ 0.030 N=5	1.17 $\pm$ 0.074 ^e N=5

ND, not detectable (<0.05 μ g/pituitary). Significant statistical differences in the AL ACTH levels were observed; ^{*}, between Ctr and Dex of 129 mice ($P<0.0001$); [†], between Ctr and Dex of B6 mice ($P<0.0001$); [‡], between 129 Ctr and B6 Ctr mice ($P<0.0001$). Significant statistical differences in the NIL ACTH levels were observed; [§], between Ctr and Dex of 129 mice ($P=0.0025$); ^{||}, between Ctr and Dex of B6 mice ($P=0.0012$); [¶], between 129 Ctr and B6 Ctr mice ($P<0.0001$); ^a, between 129 Dex and B6 Dex mice ($P<0.0001$). Significant statistical differences in the NIL α -MSH levels were observed; ^b, between Ctr and Dex of 129 mice ($P<0.0001$); ^c, between 129 Dex and B6 Dex mice ($P<0.0001$). Significant statistical differences in the NIL β -endorphin levels were observed; ^d, between Ctr and Dex of 129 mice ($P=0.0132$); ^e, between 129 Dex and B6 Dex mice ($P=0.0002$).

Figure 2 PC2 protein levels are increased when compared with their controls in Dex-treated B6 NIL, but not in Dex-treated 129 NIL (A). The NILs of both Dex-treated 129 and B6 mice contain reduced POMC levels (B). NIL lysates were analyzed by SDS-PAGE followed by western blotting using PC2 antiserum.

Dexamethasone administration decreases dopamine levels in 129 NIL and induces a prominent ER–Golgi complex in 129 melanotrophs; total D₂R mRNA levels are slightly increased in Dex-treated 129 NIL

Interestingly, total NIL surface area was increased in Dex-treated 129 NIL (1.5-fold), indicating that 1 week of Dex administration causes melanotroph proliferation in 129 mice, but not in B6 mice (Fig. 3A). Since dopamine has a key role in the control of cell proliferation and in the maintenance of the melanotroph phenotype (Saiardi & Borelli 1998), we determined total dopamine levels and D₂R mRNA densities (grains per μm^2) in the NILs of Dex-treated 129 and B6 mice using RIA and *in situ* hybridization respectively. In addition, we studied the morphological effect of Dex at the ultrastructural level using electron microscopy.

Basal dopamine levels in the NILs of 129 mice were significantly higher than those of B6 NILs (Fig. 3B), possibly due to the different sizes of the tissue in each strain (Fig. 3A). Dex treatment produced a lower dopaminergic tone in 129 NIL (1.3-fold), but not in B6 NIL (Fig. 3B), indicating differential steroid control. However, the dopamine level in the AL of both the strains of mice was unaltered following Dex treatment (data not shown). Since the synthesis and release of α -MSH from the NIL are under inhibitory control by dopamine (Goudreau *et al.* 1995), these decreased dopamine levels in the Dex-treated 129 NIL would be expected to result in enhanced release and synthesis of

Figure 3 NIL dopamine levels are decreased in Dex-treated 129 mice. (A) The area volume (*significant differences between 129 and B6 NILs, $P < 0.0001$; †significant differences between Ctr and Dex-treated 129 NILs, $P < 0.0298$). (B) NIL D₂R densities (grains per area) of Dex-treated 129 animals were decreased over their WT controls (* $P < 0.0001$; $n = 10$); however, total D₂R mRNA levels in Dex-treated 129 NIL were slightly increased. (C) D₂R density (*significant differences between Ctr and Dex-treated 129 NILs, $P = 0.0556$). (D) *In situ* hybridization of D₂R mRNA.

α -MSH; this was clearly not the case (Table 1). However, melanotrophs in Dex-treated 129 mice, but not in B6 mice, contained a prominent endoplasmic reticulum and Golgi complex, a phenotype associated with dopaminergic loss (Fig. 4; Gary & Chronwall 1992, Chronwall *et al.* 1996).

Dex treatment resulted in a trend ($P = 0.056$) toward decreased D₂R mRNA densities in 129 NIL, but not in B6 NIL (Fig. 3C). However, when the values of D₂R mRNA densities are corrected for the larger sizes of Dex-treated 129 NILs (1.5-fold), increased total D₂R mRNA contents (1.2-fold) are observed. This result is consistent with our finding of decreased dopamine levels in Dex-treated 129 NIL (Fig. 3B). *In vivo* chronic treatment with D₂R antagonists is also known to increase D₂R mRNA expression (Autelitano *et al.* 1989).

Figure 4 Dex-treated 129 melanotrophs show a prominent endoplasmic reticulum and Golgi complex (upper panel), while Dex-treated B6 melanotrophs do not differ from untreated controls at the ultrastructural level (lower panel). These data confirm the downregulation of dopaminergic control known to be associated with secretory membrane proliferation in 129 mice, but not in B6 mice. Inset, Image enlarged and enhanced by Corel Photo-Paint software.

Dexamethasone treatment of 129 animals results in considerably decreased glucose levels

We previously found that 7B2 nulls on the 129 background exhibit severe hypoglycemia; this contributes greatly to

lethality (Sarac *et al.* 2002). In the work reported here, we observed that approximately 15% of the Dex-treated 129 mice unexpectedly died at about day 5, while all B6 mice always survived Dex treatment. We determined serum glucose,

Figure 5 Dex-treated 129 animals exhibit lowered blood sugar levels and are hyperinsulinemic and hyperglucagonemic compared with their WT controls (Ctr). (A) Glucose levels (*significant differences between Ctr and Dex-treated 129 mice, $P < 0.0001$). (B) Insulin levels (†significant differences between Ctr and Dex-treated 129 mice, $P < 0.0001$; ‡significant differences between Ctr and Dex-treated B6 mice, $P < 0.0001$). (C) Glucagon levels (§significant differences between Ctr and Dex-treated 129 mice, $P = 0.0071$; ||significant differences between Ctr and Dex-treated B6 mice, $P < 0.0001$; $n \geq 10$).

insulin, and glucagon levels in the surviving mice. Dex-treated 129 mice exhibited lowered blood sugar levels and strikingly elevated insulin levels, but only slightly increased levels of glucagon (Fig. 5). This result suggests that the lethality observed in Dex-treated 129 mice could occur, at least in part, due to considerably decreased glucose levels. In contrast, Dex treatment of B6 mice resulted in highly increased insulin and glucagon levels (Fig. 5); however, this did not result in lowered blood sugar levels, suggesting greater resilience and/or compensatory mechanisms in this strain.

Discussion

We have previously reported that the Cushing's disease-like symptoms generated by intermediate lobe ACTH hypersecretion and adrenal hypercorticism in 7B2 nulls are heavily influenced by background (Peinado *et al.* 2005). In addition, we found that hypercorticism is directly involved in the lethal phenotype of the 7B2 null, as adrenalectomy rescues this null from early death (Laurent *et al.* 2002). Since we observed a 3.5-fold drop in pituitary dopamine (an inhibitory transmitter controlling NIL peptide release) in 129 7B2 null mice, but not in B6 7B2 nulls, and

since dopamine levels return to normal in adrenalectomized animals (Laurent *et al.* 2002), we hypothesized that steroids could somehow be involved in the dopaminergic disinhibition phenomenon, generating increased intermediate lobe peptide release. Further, this effect would have to occur in a strain-specific manner, since it does not occur in B6 7B2 nulls. This led us to the present investigation of potential differential strain effects of glucocorticoids on the HPA axis.

Dex treatment results in differential effects on pituitary dopamine and POMC processing in 129 and B6 mice

We observed that chronic Dex treatment indeed reduces pituitary dopamine in 129 mice, but not in B6 mice, upholding our previous results on strain-specific steroidal control of pituitary dopamine levels (Laurent *et al.* 2002). Interestingly, we observed a distinct enlargement of the NILs in Dex-treated 129 mice, accompanied by extensive rough endoplasmic reticulum and a prominent Golgi apparatus in melanotrophs. Earlier studies by others had shown that administration of D_2R antagonists *in vivo* results in proliferation of melanotrophs. D_2R -deficient mice on the mixed 129/Sv $\times$ C57BL/6 genetic background, with a 75% contribution of C57BL/6 background, show enhanced

melanotroph numbers (Saiardi & Borelli 1998), and removal of dopaminergic axons by the administration of 6-hydroxydopamine results in an increase in melanotroph proliferation in rat pituitary intermediate lobe (Gary & Chronwall 1992), linking downregulation of dopaminergic control to melanotroph proliferation. Therefore, the melanotroph proliferation exhibited by Dex-treated 129 mice is likely to be caused by the decrease in dopamine. It is interesting that these effects do not occur in B6 mice, a strain resistant to Dex-induced changes in dopamine.

Since dopamine is an inhibitory transmitter in the NIL, we hypothesized that the decreased dopaminergic tone generated by Dex treatment of 129 mice would result in increased circulating POMC-derived peptide levels; however, Dex treatment of 129 mice resulted in significantly decreased rather than increased circulating α -MSH, the intermediate lobe ACTH-derived peptide product in wild-type mice. Since Dex-treated 129 mice also exhibited reduced NIL POMC, α -MSH, and β -endorphin levels, clearly Dex affects POMC synthesis in 129 NIL; if there is a Dex effect on NIL release, it is overshadowed by Dex effects on synthesis. Previous studies have shown that Dex treatment results in a reduction in NIL POMC mRNA in both intact and adrenalectomized rats (Roberts *et al.* 1982, Schachter *et al.* 1982). It has also been found that long-term Dex treatment of rats abolishes the haloperidol-induced increase in β -endorphin, but further increases the haloperidol-induced rise in POMC mRNA (Autelitano *et al.* 1987), supporting the idea that circulating glucocorticoids can have complex and potent effects on NIL POMC expression and secretion.

The mechanism by which glucocorticoids can affect NIL POMC is not well understood. It has been demonstrated that the expression of GR in adult rat melanotropic cells is under negative dopaminergic control (Antakly *et al.* 1987). Schimchowitsch *et al.* (1994) reported the presence of functional GR, which negatively regulates melanotropic activity in the rabbit pituitary intermediate lobe. We performed western blotting to observe potentially induced changes in pituitary GR expression levels, but it proved impossible to detect any GR bands (data not shown). *In situ* hybridization analysis using PCR-amplified GR signals is needed to elucidate whether the reduction of dopamine levels has an effect on GR mRNA expression. In conclusion, since we could not recapitulate the NIL hypersecretion phenomenon using Dex treatment of wild-type mice, but did observe the expected dopamine decrease, the reduction in dopamine in the 129 7B2 null is unlikely to represent a primary cause of its NIL ACTH hypersecretion.

Dex treatment of B6 mice resulted in different cellular and circulating profiles of POMC-derived peptides when compared with 129 mice. While we also observed reduced NIL ACTH, α -MSH, and β -endorphin in this strain, dopamine levels were unaltered, and an increase in circulating α -MSH was found. Interestingly, Dex treatment also resulted in significantly increased PC2 levels in the B6 (but not in the 129) NIL; since this enzyme is responsible for processing

ACTH to α -MSH, this increase may directly explain the decreased tissue ACTH and increased circulating MSH levels. Other studies have shown that Dex treatment increases PC2 expression in the ocular ciliary epithelium (Ortego *et al.* 2002) and the endocrine cell line rMTC 6-23 (Barbero & Kitabgi 1999). In contrast, the 129 NIL seemed to be fairly resistant to PC2 induction by Dex, indicating strain-specific differences in control of PC2 expression. In summary, the ability of Dex to control the synthesis and release of POMC-derived peptides in either the 129 or B6 NIL appears to occur directly through effects on POMC and PC2 expression rather than via dopaminergic mechanisms.

Dex treatment uncovers strain differences in glucose regulation

In addition to strain-dependent effects of Dex on pituitary peptides, we observed differences in glucose regulation. For example, Dex-treated 129 animals exhibited lowered blood sugar levels and highly increased insulin levels. In contrast, Dex-treated B6 animals, while also showing considerably increased insulin levels, had slightly increased glucose levels relative to controls. Recently, Goren *et al.* (2004) reported that male C57BL/6 animals are susceptible to glucose intolerance, which is associated with impaired insulin sensitivity in liver and fat tissues, whereas the liver and fat tissues from 129 $\times$ 1 animals exhibit greater insulin sensitivity than the same tissues from other strains. Therefore, the Dex-induced decrease in glucose levels in 129 mice might be due to strain-dependent insulin hypersensitivity, a hypothesis which requires testing through additional experiments. In addition, we observed that although insulin-induced hypoglycemia induces a rapid increase in plasma levels of hormones (glucagon, epinephrine, growth hormone, and cortisol) related to the glucose counterregulatory response (Cryer 1993), the increase in insulin was significantly higher in Dex-treated 129 mice when compared with Dex-treated B6 mice, while glucagon levels were lower in 129 mice after Dex treatment than in B6 mice. The lowered blood sugar levels seen in Dex-treated 129 mice support our previous data showing that older 7B2 nulls on the 129 background exhibit severe hypoglycemia, which contributes greatly to lethality (Sarac *et al.* 2002).

In conclusion, our study suggests that although both strains of mice can expand their insulin supply in response to increased Dex, only 129 mice exhibit glucocorticoid hypersensitivity, and these mice may consequently be more susceptible to the increased functional stress imposed by chronic corticosteronemia, resulting in their much greater morbidity when challenged with Dex administration. The exaggerated Dex response in 129 mice appears to be due to the failure of normal regulatory mechanisms, which modulate hormonal secretion and/or deficits in compensatory responses to impaired glucose homeostasis. In addition to the data showing that Dex-treated 129 NILs exhibit blunted inhibitory dopaminergic control of POMC expression and secretion, our results imply that the 129 mouse is already

predisposed to Cushing's disease and Dex treatment induces further iatrogenic Cushing's disease. Therefore, we clearly demonstrate here that the lethal phenotype observed in 129 7B2 nulls (Laurent *et al.* 2002), but not in B6 7B2 nulls (Peinado *et al.* 2005), is caused by strain-dependent differences in steroid sensitivity. These considerable strain differences between 129 and B6 mice may provide a promising model system for studying steroidal effects on the HPA axis, and must certainly be taken into account when comparing the hormonal status of nulls constructed on different backgrounds.

Acknowledgements

We thank Gregory Hubbard and Jan Dufréne for assistance with animal handling. This work was supported by grants from NIDDK 49703 to I L, DA-08622 to J E P, and CIHR MOP-79283 and MOP-57870 to R D. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Antakly T, Mercille S & Cote JP 1987 Tissue-specific dopaminergic regulation of the glucocorticoid receptor in the rat pituitary. *Endocrinology* **120** 1558–1562.
- Autelitano DJ, Clements JA, Nikolaidis I, Canny BJ & Funder JW 1987 Concomitant dopaminergic and glucocorticoid control of pituitary proopiomelanocortin messenger ribonucleic acid and beta-endorphin levels. *Endocrinology* **121** 1689–1696.
- Autelitano DJ, Snyder L, Sealfon SC & Roberts JL 1989 Dopamine D2-receptor messenger RNA is differentially regulated by dopaminergic agents in rat anterior and neurointermediate pituitary. *Molecular and Cellular Endocrinology* **67** 101–105.
- Barbero P & Kitabgi P 1999 Protein 7B2 is essential for the targeting and activation of PC2 into the regulated secretory pathway of rMTC 6–23 cells. *Biochemical and Biophysical Research Communications* **257** 473–479.
- Beaulieu M, Goldman ME, Miyazaki K, Frey EA, Eskay RL, Kebabian JW & Cote TE 1984 Bromocriptine-induced changes in the biochemistry, physiology, and histology of the intermediate lobe of the rat pituitary gland. *Endocrinology* **114** 1871–1884.
- Benjannet S, Rondeau N, Day R, Chretien M & Seidah NG 1991 PC1 and PC2 are proprotein convertases capable of cleaving proopiomelanocortin at distinct pairs of basic residues. *PNAS* **88** 3564–3568.
- Bennett DL, Bailyes EM, Nielsen E, Guest PC, Rutherford NG, Arden SD & Hutton JC 1992 Identification of the type 2 proinsulin processing endopeptidase as PC2, a member of the eukaryotic subtilisin family. *Journal of Biological Chemistry* **267** 15229–15236.
- Chen CL, Dionne FT & Roberts JL 1983 Regulation of the pro-opiomelanocortin mRNA levels in rat pituitary by dopaminergic compounds. *PNAS* **80** 2211–2215.
- Chronwall BM, Millington WR, Griffen WS, Unnerstall JR & O'Donohue TL 1987 Histological evaluation of the dopaminergic regulation of proopiomelanocortin gene expression in the intermediate lobe of the rat pituitary, involving *in situ* hybridization and [3H]thymidine uptake. *Endocrinology* **120** 1201–1211.
- Chronwall BM, Sands SA, Dickerson DS, Sibley DR & Gary KA 1996 Melanotrope dopamine D2 receptor isoform expression in the developing rat pituitary. *International Journal of Developmental Neuroscience* **14** 77–86.
- Crabbe JC Jr, Allen RG, Gaudette ND, Young ER, Kosobud A & Stack J 1981 Strain differences in pituitary beta-endorphin and ACTH content in inbred mice. *Brain Research* **219** 219–223.
- Cryer PE 1993 Glucose counterregulation: prevention and correction of hypoglycemia in humans. *American Journal of Physiology* **264** E149–E155.
- Day R, Lemaire S, Nadeau D, Keith I & Lemaire I 1987 Changes in autacoid and neuropeptide contents of lung cells in asbestos-induced pulmonary fibrosis. *American Review of Respiratory Disease* **136** 908–915.
- Day R, Schafer MK, Watson SJ, Chretien M & Seidah NG 1992 Distribution and regulation of the prohormone convertases PC1 and PC2 in the rat pituitary. *Molecular Endocrinology* **6** 485–497.
- Fortenberry Y, Liu J & Lindberg I 1999 The role of the 7B2 CT peptide in the inhibition of prohormone convertase 2 in endocrine cell lines. *Journal of Neurochemistry* **73** 994–1003.
- Fortenberry Y, Hwang JR, Apletalina EV & Lindberg I 2002 Functional characterization of ProSAAS: similarities and differences with 7B2. *Journal of Biological Chemistry* **277** 5175–5186.
- Furuta M, Yano H, Zhou A, Rouille Y, Holst JJ, Carroll R, Ravazzola M, Orci L, Furuta H & Steiner DF 1997 Defective prohormone processing and altered pancreatic islet morphology in mice lacking active SPC2. *PNAS* **94** 6646–6651.
- Furuta M, Carroll R, Martin S, Swift HH, Ravazzola M, Orci L & Steiner DF 1998 Incomplete processing of proinsulin to insulin accompanied by elevation of Des-31,32 proinsulin intermediates in islets of mice lacking active PC2. *Journal of Biological Chemistry* **273** 1–7.
- Gary KA & Chronwall BM 1992 The onset of dopaminergic innervation during ontogeny decreases melanotrope proliferation in the rat pituitary intermediate lobe. *International Journal of Developmental Neuroscience* **10** 131–142.
- Goren HJ, Kulkarni RN & Kahn CR 2004 Glucose homeostasis and tissue transcript content of insulin signaling intermediates in four inbred strains of mice: C57BL/6, C57BLKS/6, DBA/2, and 129X1. *Endocrinology* **145** 3307–3323.
- Goudreau JL, Falls WM, Lookingland KJ & Moore KE 1995 Periventricular-hypophysial dopaminergic neurons innervate the intermediate but not the neural lobe of the rat pituitary gland. *Neuroendocrinology* **62** 147–154.
- Hunter WM & Greenwood FC 1962 Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature* **194** 495–496.
- Laurent V, Kimble A, Peng B, Zhu P, Pintar JE, Steiner DF & Lindberg I 2002 Mortality in 7B2 null mice can be rescued by adrenalectomy: involvement of dopamine in ACTH hypersecretion. *PNAS* **99** 3087–3092.
- Lundblad JR & Roberts JL 1988 Regulation of proopiomelanocortin gene expression in pituitary. *International Journal of Developmental Neuroscience* **9** 135–158.
- Mains RE & Eipper BA 2000 Proopiomelanocortin synthesis and cell-specific processing. In *Handbook of Physiology, section 7: the Endocrine System*, vol IV (ch V), pp 85–101. Oxford, UK: Oxford University Press.
- Muller L & Lindberg I 1999 The cell biology of the prohormone convertases PC1 and PC2. *Progress in Nucleic Acids Research* **63** 69–108.
- Muller L, Zhu X & Lindberg I 1997 Mechanism of the facilitation of PC2 maturation by 7B2: involvement in ProPC2 transport and activation but not folding. *Journal of Cell Biology* **139** 625–638.
- Muller L, Zhu P, Juliano MA & Lindberg I 1999 A 36-residue peptide contains all of the information required for 7B2-mediated activation of prohormone convertase 2. *Journal of Biological Chemistry* **274** 21471–21477.
- Murphy BE 1991 Treatment of major depression with steroid suppressive drugs. *Journal of Steroid Biochemistry and Molecular Biology* **39** 239–244.
- Ortego J, Wollmann G & Coca-Prados M 2002 Differential regulation of gene expression of neurotensin and prohormone convertases PC1 and PC2 in the bovine ocular ciliary epithelium: possible implications on neurotensin processing. *Neuroscience Letters* **333** 49–53.
- Peinado JR, Laurent V, Lee SN, Peng BW, Pintar JE, Steiner DF & Lindberg I 2005 Strain-dependent influences on the hypothalamo-pituitary-adrenal axis profoundly affect the 7B2 and PC2 null phenotypes. *Endocrinology* **146** 3438–3444.
- Raymond V, Lepine J, Lissitzky JC, Cote J & Labrie F 1979 Parallel release of ACTH, beta-endorphin, alpha-MSH and beta-MSH-like immunoreactivities in rat anterior pituitary cells in culture. *Molecular and Cellular Endocrinology* **16** 113–122.

- Roberts JL, Chen CL, Eberwine JH, Evinger MJ, Gee C, Herbert E & Schachter BS 1982 Glucocorticoid regulation of proopiomelanocortin gene expression in rodent pituitary. *Recent Progress in Hormone Research* **38** 227–256.
- Rouille Y, Bianchi M, Irminger JC & Halban PA 1997 Role of prohormone convertase PC2 in the processing of proglucagon to glucagon. *FEBS Letters* **413** 119–123.
- Rychter J & Stepien H 1977 Effects of pimozide and bromocriptine on the proliferation of rat pituitary pars intermedia cells. *Journal of Endocrinology* **75** 443–444.
- Saiardi A & Borelli E 1998 Absence of dopaminergic control on melanotrophs leads to Cushing's-like syndrome in mice. *Molecular Endocrinology* **12** 1133–1139.
- Sarac MS, Zieske AW & Lindberg I 2002 The lethal form of Cushing's in 7B2 null mice is caused by multiple metabolic and hormonal abnormalities. *Endocrinology* **143** 2324–2332.
- Schachter BS, Johnson LK, Baxter JD & Roberts JL 1982 Differential regulation by glucocorticoids of proopiomelanocortin mRNA levels in the anterior and intermediate lobes of the rat pituitary. *Endocrinology* **110** 1442–1444.
- Schimchowitsch S, Plante M, Kienlen P, Felix JM, Koch B & Stoeckel ME 1994 Glucocorticoids, but not dopamine, negatively regulate the melanotrophic activity of the rabbit pituitary intermediate lobe. *Journal of Neuroendocrinology* **6** 385–390.
- Seidah NG, Gaspar L, Mion P, Marcinkiewicz M, Mbikay M & Chretien M 1990 cDNA sequence of two distinct pituitary proteins homologous to Kex2 and furin gene products: tissue-specific mRNAs encoding candidates for pro- hormone processing proteinases. *DNA and Cell Biology* **9** 789.
- Seidah NG, Chretien M & Day R 1994 The family of subtilisin/kexin like pro-protein and pro-hormone convertases: divergent or shared functions. *Biochimie* **76** 197–209.
- Shen FS, Seidah NG & Lindberg I 1993 Biosynthesis of the prohormone convertase PC2 in Chinese hamster ovary cells and in rat insulinoma cells. *Journal of Biological Chemistry* **268** 24910–24915.
- Snyder SE, Pintar JE & Salton SR 1998 Developmental expression of VGF mRNA in the prenatal and postnatal rat. *Journal of Comparative Neurology* **394** 64–90.
- Suda T, Tomori N, Tozawa F, Mouri T, Demura H & Shizume K 1984 Effect of dexamethasone on immunoreactive corticotropin-releasing factor in the rat median eminence and intermediate-posterior pituitary. *Endocrinology* **114** 851–854.
- Vale W, Spiess J, Rivier C & Rivier J 1981 Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science* **213** 1394–1397.
- Vindrola O & Lindberg I 1992 Biosynthesis of the prohormone convertase mPC1 in AtT-20 cells. *Molecular Endocrinology* **6** 1088–1094.
- Westphal CH, Muller L, Zhou A, Zhu X, Bonner-Weir S, Schambelan M, Steiner DF, Lindberg I & Leder P 1999 The neuroendocrine protein 7B2 is required for peptide hormone processing *in vivo* and provides a novel mechanism for pituitary Cushing's disease. *Cell* **96** 689–700.
- Zhu X & Lindberg I 1995 7B2 facilitates the maturation of proPC2 in neuroendocrine cells and is required for the expression of enzymatic activity. *Journal of Cell Biology* **129** 1641–1650.

Received in final form 30 November 2006

Accepted 6 December 2006

Made available online as an Accepted Preprint

27 December 2006