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Genetic Disruption of Dopamine Production Results in Pituitary Adenomas and Severe Prolactinemia

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Canine adenovirus · Dopamine deficiency · Lactotroph · Pituitary · Prolactin · Prolactinoma · Tuberoinfundibular · Viral gene transfer

Abstract
Background: Dopamine release from tuberoinfundibular dopamine neurons into the median eminence activates dopamine-D2 receptors in the pituitary gland where it inhibits its lactotroph function. ***Methods:*** We have previously described genetic dopamine-deficient mouse models which lack the ability to synthesize dopamine. Because these animals require daily treatment with 3,4-L-dihydroxyphenylalanine (L-dopa) to survive, it has not been possible to examine the consequences of chronic loss of dopamine on pituitary physiology. We use viral-mediated gene transfer to selectively restore dopamine to the dorsal striatum of dopamine-deficient mice which allows the mice to survive without L-dopa. ***Results:*** We find that mice chronically lacking tuberoinfundibular dopamine secrete large amounts of prolactin due to the development of severely enlarged pituitaries composed principally of hyperplastic hypertrophic lactotrophs and multifocal prolactinomas. In addition, these mice have elevated serum growth hormone levels and aged males develop hypertrophy of the seminal vesicles. ***Conclusion:*** Our observations are consistent with the hypothesis that hypothalamic dopamine is a critical inhibitor of lactotroph proliferation and suggest additional roles for dopamine in the regulation of pituitary function.

Introduction

Prolactin (PRL) release from the anterior pituitary is unique in that the principal regulatory factor is the neurotransmitter dopamine which provides an inhibitory signal to lactotrophs via activation of the dopamine-D2 receptor (D2R). Dopamine reaches the pituitary primarily through release from neurosecretory tuberoinfundibular dopamine (TIDA) neurons located in the dorsomedial arcuate nucleus. Low serum PRL is maintained by tonic dopamine release into the median eminence where portal blood vessels carry it to the anterior pituitary [1]. Elevation of serum PRL is mediated primarily by inhibition of TIDA neurons but may also be controlled by pituitary-derived PRL releasing factors [2].

The principal therapy for human pituitary adenomas is treatment with D2R agonists, which effectively lower serum PRL and reduces tumor size [1]. Furthermore, genetic disruption of the gene encoding D2R leads to ele-

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vated serum PRL and progressive lactotroph proliferation [3, 4]. Together, these data provide compelling evidence that dopamine is a principal inhibitor of pituitary lactotroph proliferation and PRL release. On the other hand, the consequence of chronic dopamine deficiency on pituitary function has not been directly assessed. Genetically modified mice lacking *Tyrosine hydroxylase (Th)* specifically in dopamine neurons require daily treatment with 3,4 L-dihydroxyphenylalanine (L-dopa) to consume sufficient food for survival [5]. Because these dopamine-deficient (DD) mice are dopamine depleted ~16 h per day but dopamine repleted ~8 h per day, interpretation of any deficits in pituitary function (or lack thereof) would be difficult.

However, we recently created a dopamine-deficient, floxed-stop (DDfs) mouse model with a conditionally inactive *Th* allele. DDfs mice share many characteristics with the original DD line but allow for viral-driven expression of Cre recombinase to permanently restore endogenous *Th* gene expression to midbrain dopamine neurons [6]. Selective restoration of dopamine signaling to the nigrostriatal circuit is sufficient to restore feeding and locomotor behaviors independent of L-dopa treatment. Here, we use this model to examine the consequences of dopamine deficiency outside of the nigrostriatal circuit on pituitary function.

Materials and Methods

Generation of vrDDfs Mice

Dopamine-deficient floxed-stop (DDfs) mice were generated as described [6]. Briefly, *Th* floxed-stop ($Th^{fs/+}$) mice with a floxed neomycin resistance gene that disrupts *Th* gene expression were created by gene targeting. DDfs ($Th^{fs/fs}$; $Dbh^{Th/+}$) mice carry two conditionally inactive *Th* alleles, one intact *Dopamine β-hydroxylase (Dbh)* allele (Dbh^{+}), and one *Dbh* allele with a targeted insertion of the *Th* gene (Dbh^{Th}). Control animals carry at least one intact *Th* allele and one intact *Dbh* allele. Mice were maintained on a mixed C57BL/6 × 129/SvEv/S4 genetic background (~90% C57BL/6). All mice were housed under a 12:12 light/dark cycle in a temperature-controlled environment with food (5LJ5; PMI Nutrition, St. Louis, Mo., USA) and water available ad libitum. Females used in these studies were randomly cycling. All mice were treated in accordance with guidelines established by the National Institute of Health and the University of Washington Animal Care Committee.

A recombinant CAV-2 vector engineered to express Cre-recombinase driven by the cytomegalovirus promoter was generated and titered as described [7]. The vector preparation had a titer of 6×10^{12} physical particles/ml. Bilateral injections of 0.5–1.0 μl CAVCre into the central region of the caudate putamen (CPu) (coordinates in mm: +0.80 anterior to Bregma, +2.00 and –2.00 lateral to midline; 3.60 ventral to skull surface) were performed on anesthetized (ketamine, xylazine, acepromazine) 3- to 5-month-old DDfs and control mice as described [6, 8]. DDfs mice were removed from L-dopa treatment ~1 week after viral injection and thereafter referred to as virally rescued (vrDDfs).

Histology

Anesthetized mice were perfused transcardially with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in PBS. Brains and pituitaries were extracted and postfixed in PFA overnight. Brains were cryoprotected in 30% sucrose, and frozen in super-chilled isopentane. Free-floating coronal sections (30–40 μm) were immunostained using rabbit anti-TH (1:2,000; Chemicon, Temecula, Calif., USA). Immunofluorescence was revealed using CY2/3-labeled donkey secondary antibody (1:200; Jackson Immunoresearch Laboratories, West Grove, Pa., USA). Sections were mounted on slides, coverslips applied, and photographed. Following fixation, pituitaries were transferred through ethanol (70, 95, 100%) and xylene rinses. Pituitaries were embedded in paraffin (Polysciences Inc., Warrington, Pa., USA) and sections (8 μm) were mounted on slides. Slides were rehydrated, rinsed with 0.3–0.5% Triton-X-100 in PBS, blocked with 2.5–10% normal donkey serum, and one of the following antibodies was applied: rabbit anti-PRL (1:500; National Hormone & Peptide Program, Torrance, Calif., USA), rabbit anti-growth hormone (GH) (1:150; National Hormone & Peptide Program), sheep anti-α-melanocyte-stimulating hormone (αMSH) (1:500; Chemicon) or goat anti-β-TSH (1:20; Santa Cruz Biotechnology, Santa Cruz, Calif., USA). Immunofluorescence was revealed using CY2/3-labeled donkey secondary antibody (1:200–1:1,000; Jackson Immunoresearch Laboratories), slides were dehydrated, coverslips applied, and photographed using standard immunofluorescence or confocal microscopy. DAPI, when added, was applied following immunostaining at a concentration of 0.1 μg/ml. Gomori's stain for reticulin fibers was performed as described elsewhere [9].

Sera Measures

Blood was collected from the saphenous vein of mice ~12 months following viral rescue (15–17 months of age). The blood was kept on ice until centrifuged at 4°C, the plasma was removed, and the sera frozen at –80°C. Sera measurements of PRL, GH, luteinizing hormone (LH), and testosterone were performed by radioimmunoassay under the supervision of A.F. Parlow at NIDDK-NHPP.

Results

Restoration of Dopamine to the Dorsal Striatum, but Not Hypothalamus, of DD Mice

Stereotaxic injection of CAVCre into the CPu leads to expression of Cre recombinase in the midbrain dopamine neurons of the substantia nigra pars compacta (SNc) that project there; resulting in excision of the stop cassette from the first intron of the *Th* gene thereby permitting expression of endogenous *Th* [6, 10]. These vrDDfs mice are able to feed and survive indefinitely, yet their ability to produce dopamine in other dopamine systems remains

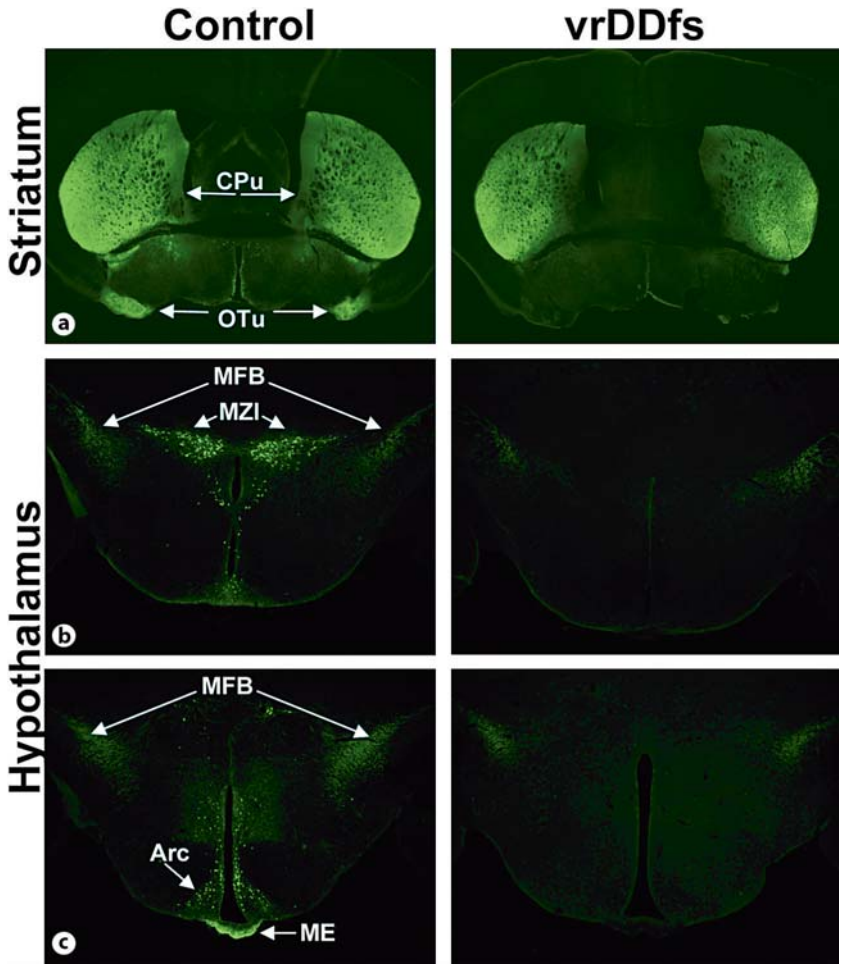

Fig. 1. TH immunostaining was absent from the hypothalamus of vrDDfs mice. Coronal sections through the dorsal striatum of control (left) and vrDDfs (right) mice (**a**). Coronal sections through the hypothalamus including the medial zona incerta (ZI) (**b**) or arcuate nucleus (Arc) and median eminence (ME) (**c**). Notice the absence of TH staining in the hypothalamic nuclei of the vrDDfs mice, although TH signal can be observed in the dopamine terminals in the caudate putamen (CPu) and the fiber tracts of the medial forebrain bundle (MFB). OTu = Olfactory tubercle.

disrupted. Indeed, TH immunoreactivity was observed throughout a large portion of the dorsal striatum of vrDDfs mice (fig. 1a). However, no TH-positive cells were ever observed in the hypothalamic dopamine cell groups of vrDDfs mice (fig. 1b, c).

vrDDfs Mice Develop Lactotroph Hyperplasia and Prolactinomas

Approximately 12 months following viral injection (and removal from L-dopa), vrDDfs and littermate control mice were sacrificed and their pituitaries were examined. At this age, all vrDDfs mice ($n > 12$) had enlarged pituitaries relative to controls (fig. 2). However, the effect was sexually dimorphic such that the pituitaries of female vrDDfs mice were larger and hemorrhagic compared to males (fig. 2a, b). The two-dimensional surface area of the pituitaries was ~2 fold larger in male and ~5 fold larger in female vrDDfs relative to controls, although these values represent an underestimate as expansion occurred in three dimensions (fig. 2c, d). Several animals examined prior to 8 months following viral rescue had no obvious increase in pituitary size compared to controls, suggesting that most of the growth occurs after 8 months of dopamine deficiency.

Light microscopic and immunohistochemical studies of the pituitary glands from vrDDfs mice at 12 months of age demonstrated diffuse lactotroph hyperplasia with multiple microadenomas, particularly in females (fig. 3). The microarchitecture, normal periacinar pattern of reticulin staining, and mixture or acidophils, basophils, and chromophobes were preserved in many areas (fig. 3b), although a subset of chromophobes (presumed lactotrophs) were individually larger and collectively more numerous than in control animals. Dilated blood-filled spaces (peliosis) separated cords of heterogeneous endocrine cells in some areas (fig. 2d, f). In addition, discrete reticulin-free nodules (adenomas) composed entirely of enlarged cells with pale cytoplasms were present

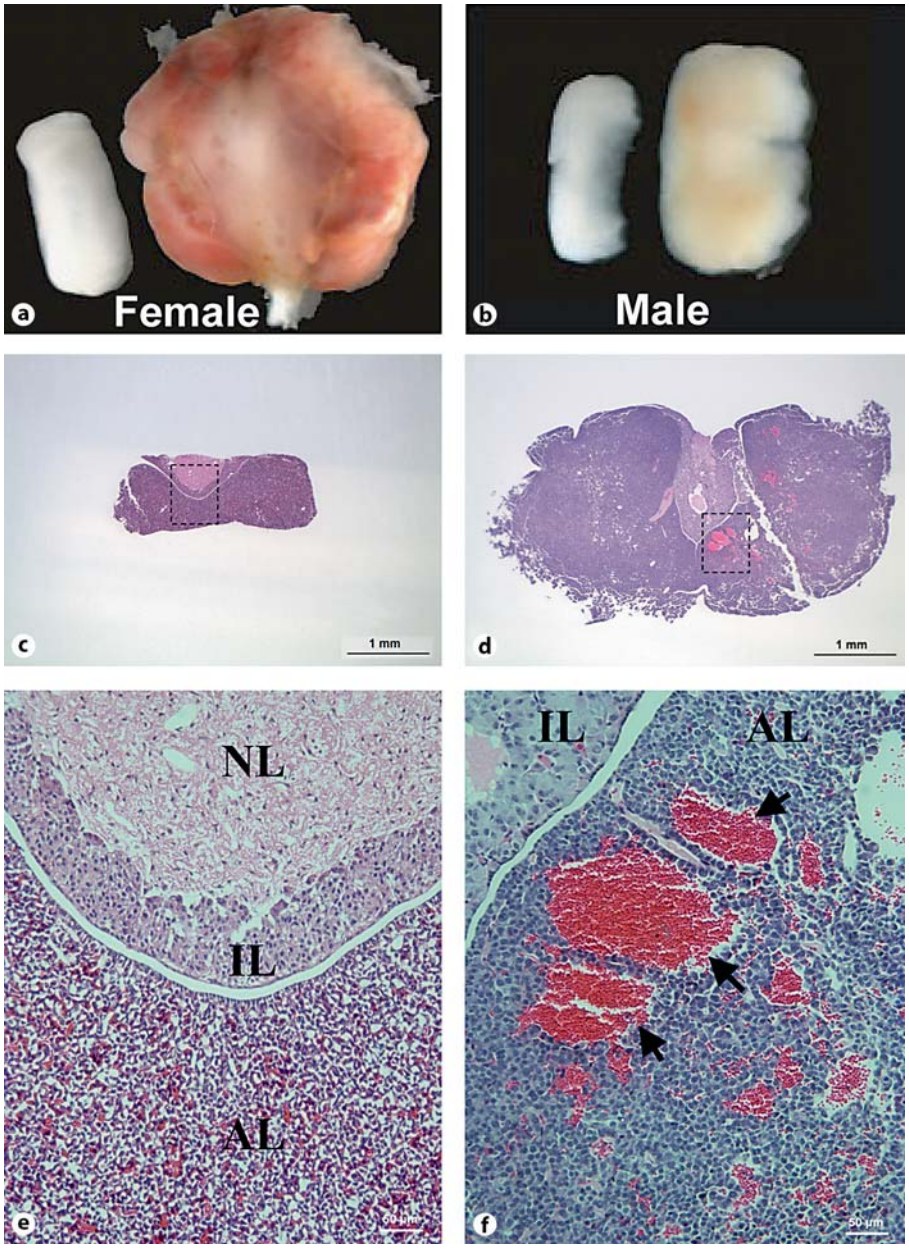

Fig. 2. Pituitary hyperplasia in vrDDfs mice. Pituitaries from control (left) and vrDDfs (right) female (**a**) and male (**b**) mice ~12 months following viral rescue. Pituitaries of vrDDfs are enlarged compared to controls and the phenotype is more pronounced in females. HE staining of control (**c**, **e**) and vrDDfs (**d**, **f**) sections from female pituitaries. Boxes in **c** and **d** indicate approximate region of magnification in **e** and **f**, respectively. Arrows in **f** indicate peliosis. AL = Anterior lobe; NL = neural lobe; IL = intermediate lobe.

(fig. 3d, e). Nuclear pleomorphism, mitotic figures, and perinuclear lamellar inclusions characterized the adenomatous populations (fig. 3c). Immunohistochemistry (IHC) directed against PRL revealed that the majority of the hyperplastic and adenomatous cells were lactotrophs (fig. 3g, 4). Furthermore, lactotrophs of vrDDfs mice were hypertrophied compared to controls (fig. 4f). In contrast, somatotrophs (as defined by GH immunofluorescence), which are normally distributed throughout the anterior pituitary (fig. 5a, c), were excluded from large portions of the anterior pituitary of vrDDfs mice (fig. 5b, d). Thyrotrophs were present in pituitaries of both control and vrDDfs, although staining was scattered and relatively scarce, especially in vrDDfs mice (fig. 6c, d). IHC against α-MSH revealed that the intermediate lobes remained intact in vrDDfs mice, but often appeared malformed (fig. 6b). However, the shapes and sizes of the intermediate lobes were not consistent across animals.

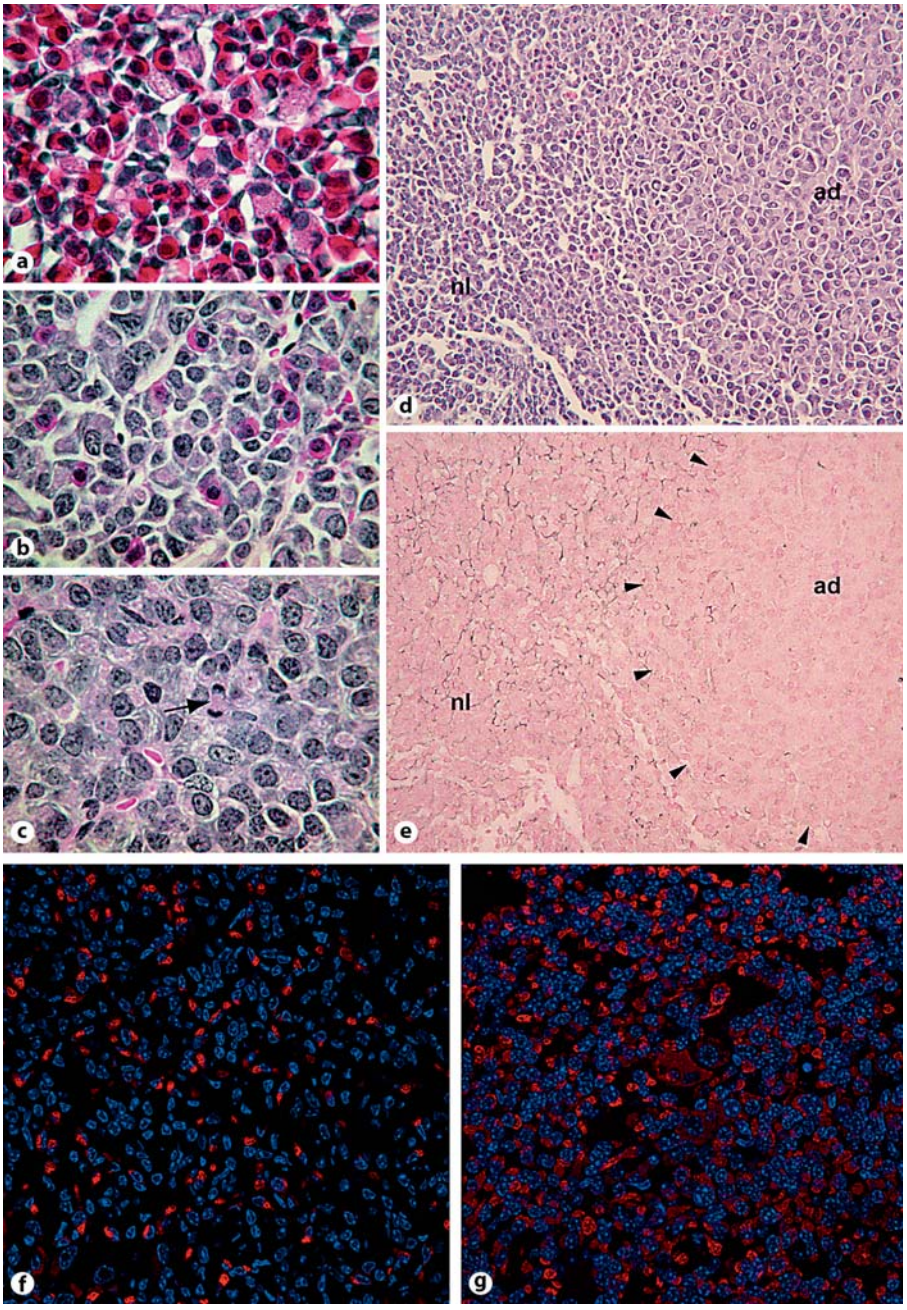

Fig. 3. Lactotroph hyperplasia and adenoma formation in female vrDDfs mice. **a** A representative microscopic field from control animal demonstrates a mixture of cell types including bright red acidophils and larger pale chromophobes. **b** A nonadenomatous area from the pituitary of a vrDDfs female shows residual trabecular architecture with scattered acidophils and hyperplasia of large putative lactotrophs with pale cytoplasms, irregular nuclei, and cytoplasmic lamellar inclusions. **c** A microadenoma from the same mouse is composed entirely of monomorphic cells with many similar cytological features to the putative lactrotrophs in **b**. A mitotic figure is present (arrow). **d** A low magnification photomicrograph shows an adenoma on the right and intact pituitary on the left. **e** A reticulin stain of an adjacent section to **d** demonstrates intact trabecular architecture in the non-adenomatous region and complete loss of black reticulin staining in the adenoma (demarcated by arrowheads). PRL (red) immunofluorescence reveals diffusely increased lactotroph density in the vrDDfs (**g**) compared to control (**f**). Nuclei are stained with DAPI (blue).

Examination of reproductive organs revealed severe seminal vesicle hypertrophy in all aged male vrDDfs ($n > 6$), but not control, mice examined (fig. 7). This was independent of testosterone or LH levels, which were unchanged across genotype (data not shown). No gross abnormalities were observed in testis, prostates, or uteri from vrDDfs compared to control mice.

vrDDfs Mice Have Elevated Serum PRL Measures

Consistent with the observation that lactotrophs preferentially proliferate in the anterior pituitary of vrDDfs mice, serum PRL levels were greatly elevated. In vrDDfs females, PRL levels were >40 fold higher than in female controls (fig. 8a). In males the increase was ~30 fold (fig. 8b). There was also ~4.5 fold increase in serum GH levels (fig. 8c).

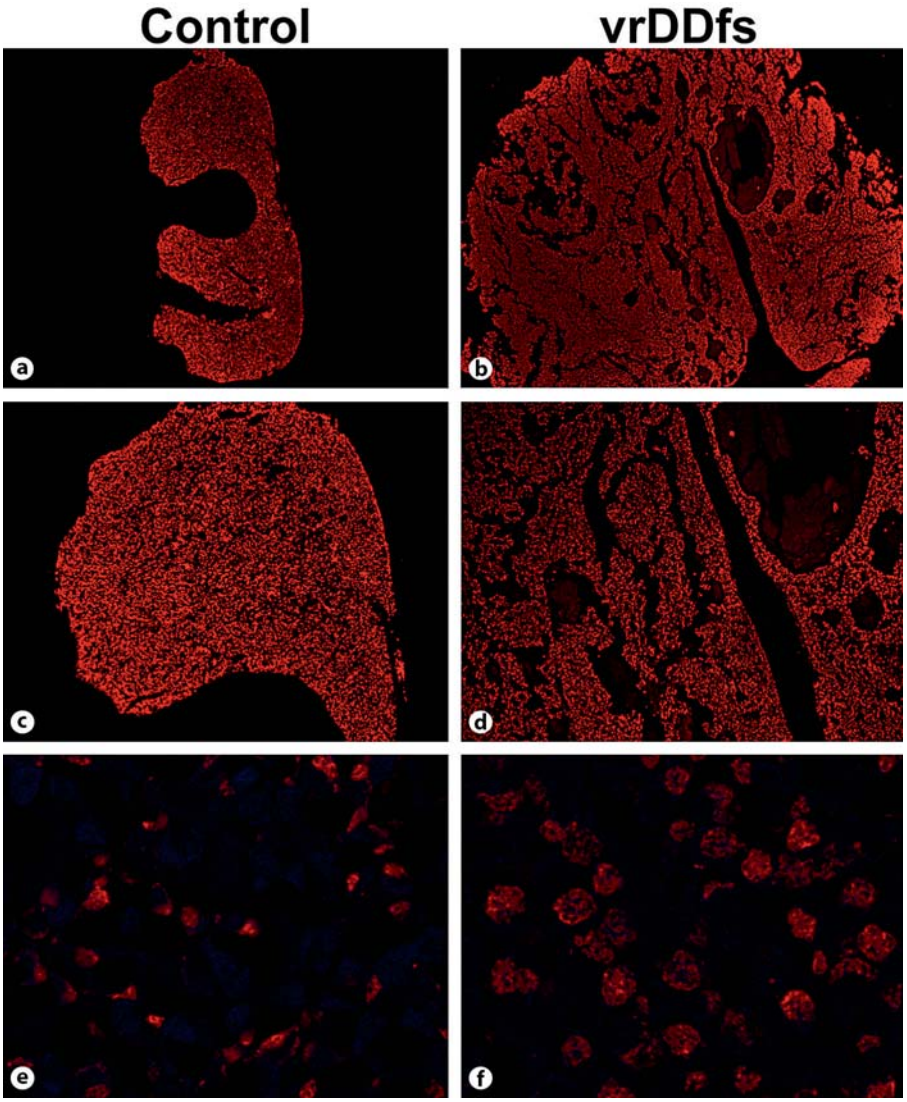

Fig. 4. PRL immunofluorescence on pituitary sections. Sections from control (**a**) and vrDDfs (**b**) female mice immunostained for PRL ~12 month following viral rescue. Notice the widespread distribution of lactotrophs. Higher magnification images of control (**c**) and vrDDfs (**d**) mice. Confocal microscopy revealing lactotrophs of vrDDfs (**f**) are hypertrophied compared to control (**e**) mice.

Discussion

Although it has been established that dopamine is an important inhibitor of lactotroph function, the consequences of hypothalamic dopamine deficiency had never been directly tested. Because DD mice require daily treatment with L-dopa for survival, their utility for experimental questions that require the chronic absence of dopamine signaling is limited. We therefore circumvented the L-dopa requirement by using viral-mediated gene transfer to restore dopamine signaling selectively to the dorsal striatum of DDfs mice [6]. This technique restores many behaviors including feeding and locomotor activity and is sufficient to allow for survival in the absence of L-dopa. We show here that vrDDfs mice lacking hypothalamic dopamine developed lactotroph hyperplasia, hypertrophy, and hyperprolactinemia. We also show evidence for neoplastic transformation in vrDDfs females, which were characterized by disruption of acinar architecture by mitotically active, lactotroph nodules that lack reticulin staining. Furthermore, GH levels were elevated compared to controls and males develop seminal vesicle hypertrophy.

The main findings of our study are consistent with the known function of dopamine as an inhibitory signal of lactotroph function [1]. Our results are also generally consistent with previous genetic mouse models in which the gene encoding D2R was disrupted and the mice dis-

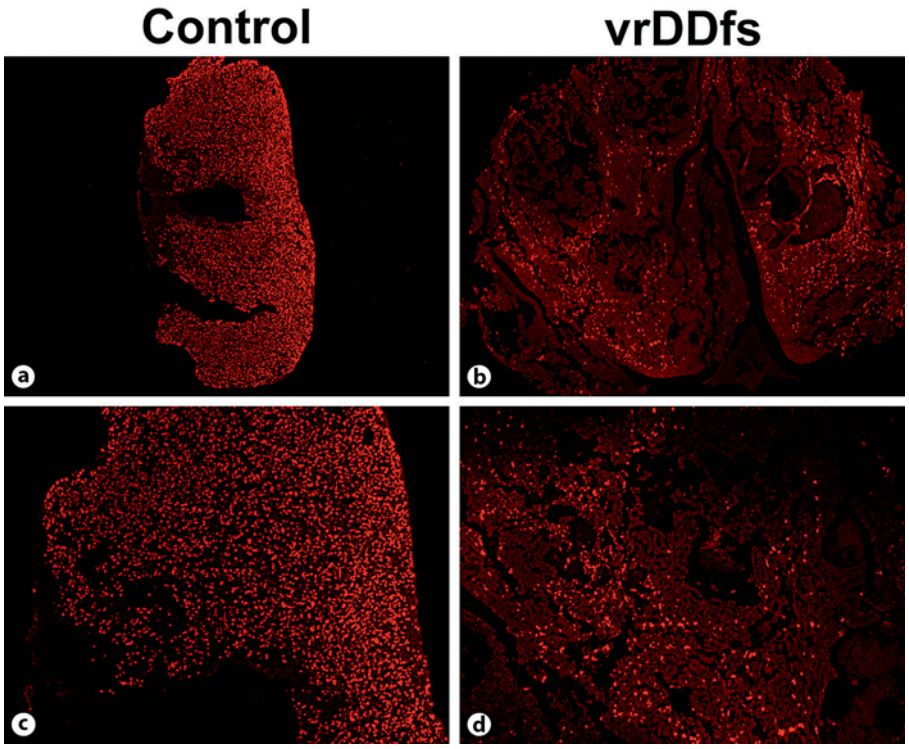

Fig. 5. GH immunofluorescence on pituitary sections. Pituitary sections stained for GH from control (**a**, **c**) and vrDDfs (**b**, **d**) female mice. The GH signal is distributed uniformly throughout the anterior pituitary in control, but is excluded from microadenomas in vrDDfs mice.

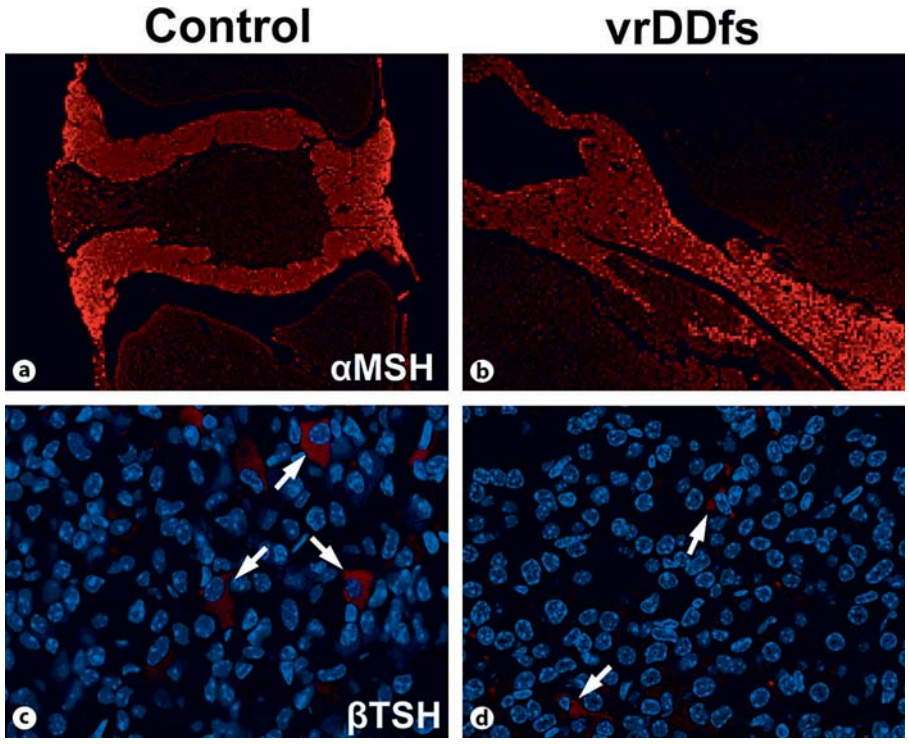

Fig. 6. Pituitary αMSH and βTSH immunofluorescence. Intermediate lobe stained for αMSH in control (**a**) and vrDDfs (**b**) female mice. The intermediate lobes were present in vrDDfs animals, but the architecture was often disrupted, presumably by the excessive growth of the anterior pituitary. Thyrotrophs expressing βTSH comprised a small population of cells in the anterior pituitary in both control (**c**) and vrDDfs (**d**) mice. Nuclei are stained with DAPI (blue).

played lactotroph hyperplasia and elevated PRL levels [3, 4, 11]. We observed larger increases (>40 fold in females) in serum PRL from vrDDfs mice compared to controls than originally reported for the D2R-knockout mice (~2- to 10-fold), but consistent with later reports which examined older animals [12]. This underscores the progressive nature of the phenotype over time [12, 13].

Our observation that pituitaries of aged female vrDDfs mice contain prolactinomas is also consistent with subsequent studies that examined older animals [4, 12, 13]. It is unlikely that the loss of dopamine is solely responsible for the initial transforming event. Instead, the absence of dopamine signaling through D2R and consequent hyperplasia may place lactotrophs at increased risk of oncogenic mutation; and the continued absence of dopamine signaling likely further promotes tumor growth.

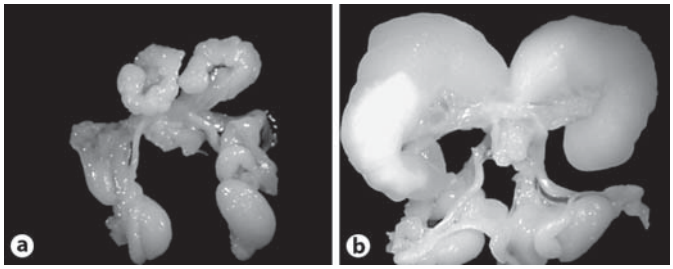

Fig. 7. Hypertrophy of seminal vesicles. Control (**a**) and vrDDfs (**b**) seminal vesicles.

Indeed, ovariectomy of D2R-knockout mice completely blocks tumor formation and greatly reduces prolactinemia [14]. This suggests that ovarian factors are required for the development of prolactinomas in the absence of D2R signaling. One such factor is estradiol, which is known to increase PRL gene expression, modulate TIDA neuron activity, and regulate lactotroph function at multiple levels [15]. Chronic estradiol treatment can lead to the formation of prolactinomas in susceptible strains of rats; formation of which can be blocked by D2R agonists [16]. Further, activation of estrogen receptors has been shown to directly activate expression of pituitary tumor transforming gene which drives angiogenesis through induction of fibroblast growth factor 2 and vascular endothelial growth factor [17, 18]. The interaction between dopamine and estradiol in the regulation of lactotroph function likely contributes significantly to the sexual dimorphism observed in the vrDDfs phenotype. Curiously, however, D2R-knockout females show altered estrous cycles and reduced serum estradiol levels [4]. In fact, D2R-knockout females have serum estradiol levels equivalent to males, suggesting that estradiol is not in fact responsible for the sex differences in the phenotypes of D2R-knockout mice – although the expression of estrogen receptors was not determined. When combined with the observation that estradiol replacement does not restore serum PRL levels of ovariectomized D2R-knockout mice to intact levels, it remains possible that other ovarian factors play important roles [14].

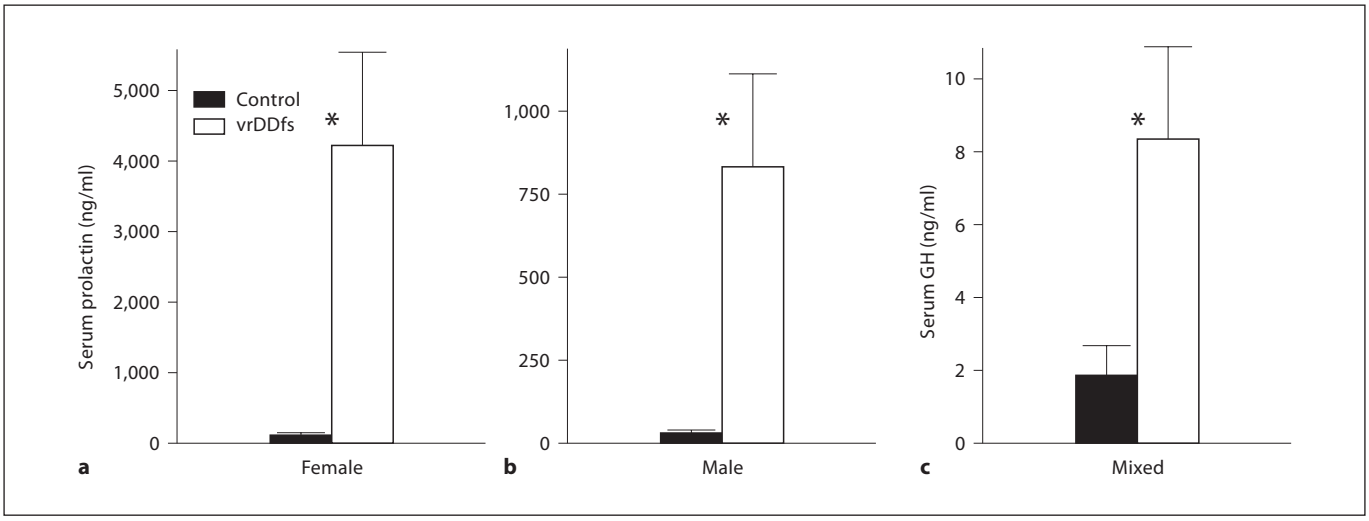

Fig. 8. Sera PRL levels in female (**a**) and male (**b**) mice. PRL levels were ~5 fold greater in females compared to males but vrDDfs mice had greatly elevated serum PRL regardless of sex. Sera GH was also elevated in vrDDfs mice (**c**). Data are presented as means ± SEM. * 2-tailed t test $p < 0.05$.

Saiardi et al. [4, 19] reported enlarged intermediate lobes in D2R-knockout mice, consistent with a role for dopamine in the control of melanotroph activity. However, Kelly et al. [3] reported no change in intermediate lobe size in D2R-knockout mice and highlighted the potential for background strain to modify this phenotype. Our DDfs colony is maintained on a mostly (~90%) C57BL/6 background; yet we observed occasional examples of enlarged intermediate lobes in the vrDDfs mice (as shown in fig. 2d). However, we are unable to decisively comment on the consequences of chronic loss of dopamine on the intermediate lobe because anterior pituitary growth markedly disrupted glandular architecture and prohibited accurate characterization of intermediate lobe size.

Our finding that serum GH levels are increased in vrDDfs mice differs from D2R-knockout mice which have decreased pituitary GH mRNA [4] and decreased serum GH levels compared to WT controls for the first 3 months of life [11]. The fact that we did not see a decrease in serum GH in vrDDfs mice is not surprising because the deficit disappears in the D2R knockout after 3 months of age and we measured GH levels between 15 and 17 months of age. We did not measure GH prior to 3 months, but following 12 months of dopamine deficiency, GH was clearly elevated; an observation that may be consistent with the reduced GH content found in pituitary tissue from hyperdopaminergic dopamine transporter (DAT)-knockout mice [20]. Collectively, these data suggest a complex role for dopamine signaling in the modulation of somatotroph activity; both a D2R-dependent component that fades after the rapid-growth period of early life and a non-D2R-dependent component which persists later in life.

The seminal vesicle hyperplasia we observed in males was surprising because it was not reported in the D2R-knockout mice, although uterine adenomyosis was observed [3]. On the other hand, the D2R-knockout males are not nearly as hyperprolactinemic as the vrDDfs males (perhaps because they were examined at a younger age). In addition, seminal vesicles express abundant levels of PRL receptor transcript [21] and PRL knockout mice have reduced seminal vesicle size [22]. Furthermore, seminal vesicle hypertrophy has been observed in other transgenic models with increased PRL and GH production [23, 24].

PRL has diverse behavioral and physiological effects in rodents. For instance, PRL-knockout mice have reduced rapid eye movement sleep [25] and PRL receptor-deficient mice have reduced body weight [26] which may be associated with changes in pancreatic islet cell function [27]. In addition, acute PRL treatment can induce feeding behavior in both rodents and doves [28, 29]. These effects of PRL on feeding and metabolism are especially interesting in light of our previous results which show that vrDDfs mice consume more food than control mice [6].

In conclusion, we directly tested the effects of chronic dopamine loss on pituitary hormone measures. Our data strongly support the hypothesis that dopamine exerts a strong inhibitory influence on lactotroph function. Indeed, the genetic approaches using the vrDDfs and D2R-knockout mice unequivocally demonstrate the essential role for dopamine signaling through D2R in the suppression of PRL release and lactotroph hyperplasia. The vrDDfs and D2R-knockout mice provide complementary models for studying the consequences of prolactinomas and hyperprolactinemia on physiology and behavior.

Acknowledgments

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