Antiproliferative Role of Dopamine: Loss of D_2 Receptors Causes Hormonal Dysfunction and Pituitary Hyperplasia

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Summary

The function of dopamine (DA) in the nervous system is paralleled by its neuroendocrine control of pituitary gland functions. Here, we document the neuroendocrine function of dopamine by studying the pituitary gland of mice lacking DA D_2 receptors (D_2R). These mice present a striking, progressive increase in lactotroph number, which ultimately leads to tumors in aged animals. Females develop tumors much earlier than males. An estrogen-mediated lactotroph proliferation cannot account for this sexual dimorphism, since D_2R-null females are hypoestrogenic and, thus, have estrogen levels similar to males. In contrast, prolactin levels are six times higher in females than in males. We show that active prolactin receptors are present in the pituitary and their expression increases in concomitance with tumor expansion. These results point to prolactin as an autocrine proliferative factor in the pituitary gland. Additionally, they demonstrate an antiproliferative function for DA regulated through D_2 receptor activation.

Introduction

The development of the pituitary gland is a remarkable process in which the temporally coordinated activity of different factors establishes cell identity (Voss and Rosenfeld, 1992; Borrelli, 1994; Andersen and Rosenfeld, 1994). In rodents, the pituitary gland is composed of the adenohypophysis, which comprises the anterior (AL) and intermediate (IL) lobes, and the neurohypophysis, which constitutes the posterior lobe (PL). Adenohypophysis development has been extensively studied, due to its relatively simple structure and low cellular complexity. The availability of cellular markers, representing the specific hormones secreted by each cell type, has allowed tracing of these cells during development. These include the gonadotrophs, which secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH); the corticotrophs, which produce adenocorticotropin (ACTH); and the melanotrophs, which produce α-melanocyte-stimulating hormone (α-MSH) and β-endorphin (β-End). The thyro-somato-mammotroph lineage deserves special mention. This lineage gives rise to the thyrotrophs, the somatotrophs, and finally the lactotrophs, which produce thyroid-stimulating hormone (TSH), growth hormone (GH), and prolactin (PRL), respectively (Slabaugh et al., 1981; Frohman, 1987; Gage et al., 1996). It has been shown that the expression of Prop-1, a paired-like homeodomain transcription factor (Sornson et al., 1996), and Pit-1/GHF1, a transcription factor of the POU homeodomain gene family, are early prerequisites for the determination of this lineage (Li et al., 1990; Rhodes et al., 1994). Subsequently, hypothalamic factors such as growth hormone-releasing hormone (GHRH) are required for the proliferation of these cells (Mayo, 1992; Lin et al., 1992; Godfrey et al., 1993). Interestingly, the proliferation and maintenance of somatotrophs and lactotrophs are controlled in opposite ways by intracellular cAMP levels. Namely, while somatotroph proliferation requires the activation of cAMP signaling, lactotroph maintenance is negatively controlled by dopamine-mediated inhibition of the same pathway (Weiner et al., 1988). A linear relationship in the development of lactotrophs from somatotrophs has been demonstrated by cell ablation studies (Behringer et al., 1988; Borrelli et al., 1989). Thus, establishment of the lactotrophs is the last differentiation step within the thyro-somato-mammotroph lineage. After birth, lactotroph proliferation appears to be regulated by estrogens (Lieberman et al., 1983). Thus, dopamine (DA) and estrogens are thought to act in an opposing fashion on lactotroph growth. Indeed, estrogen treatment of rats induces lactotroph proliferation and may lead to pituitary tumors (Elias and Weiner, 1987). Dopamine can block this effect and has also been shown to aid regression of prolactinomas, fairly frequent human pituitary tumors (Bansal et al., 1981). This suggests that a balance exists between these positive and negative signals, which is important for the maintenance of the lactotroph lineage. The mechanisms underlying estrogen stimulation of lactotroph proliferation and DA-mediated regression of prolactinomas have yet to be elucidated.

Here, we analyze the physiological relevance of dopaminergic control to the development of the pituitary gland by studying mice lacking the DA D_2 receptor (D_2R) (Baik et al., 1995). The D_2R is highly expressed in the pituitary and in the brain, where it exists in two molecular forms, D_2L and D_2S (Picetti et al., 1997). These two isoforms have been shown to couple different G-proteins (Montmayeur et al., 1993; Guiramand et al., 1995).

We show that the negative control over prolactin synthesis mediated by DA is lost in D_2R-null mice. Interestingly, these mice show a time-dependent proliferation of lactotrophs, which leads to anterior pituitary tumors in older animals. The time of onset of these tumors is delayed in males. This sexual dimorphism is due to the different levels of prolactin in the two genders. We suggest that prolactin acts as an autocrine growth factor through the activation of pituitary PRL receptors (PRL-R) (Boutin et al., 1988). Thus, DA, via the D_2Rs, appears to act not only as a key regulator of pituitary hormone secretion, but also as a master-switch directing the decision of the somato-mammotroph lineage to differentiate or proliferate.

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Results

Absence of Dopaminergic Sites in D_2R-Null Pituitaries

Dopamine function in the central nervous system is paralleled by its neuroendocrine control of pituitary functions (Frohman, 1987). The physiological effects induced by DA are mediated through the interaction with specific membrane receptors (Gingrich and Caron, 1993). In the pituitary, DA acts through receptors of the D_2-like family expressed in the lactotrophs and melanotrophs (Autelitano et al., 1989). Indeed, DA is thought to control the synthesis and release of prolactin in the AL and of hormones produced by the processing of proopiomelanocortin (POMC) in the IL (Chen et al., 1983; Cote et al., 1986; Elsholtz et al., 1991; Ben-Jonathan, 1994).

We used D_2R-null mice to establish whether the absence of D_2Rs might be compensated by overexpression of other D_2-like receptors (D_3 and D_4) previously reported in the pituitary (Herroelen et al., 1994; Valerio et al., 1994). We performed in situ ligand binding studies on pituitaries from wild-type (WT) and D_2R-null mice. Two D_2-like specific receptor antagonists were utilized, $[^{125}I]$-iodosulpride and $[^3H]$-spiperone. The first binds to D_2R and D_3R, while the second binds to D_2R and D_4R with similar affinities (Seeman and Van Tol, 1994). In situ binding analysis using $[^{125}I]$-iodosulpride, at a final concentration of 0.3 nM, reveals a strong labeling of the AL and IL of WT mice pituitaries (Figure 1, WT). In contrast, no labeling is detected in the pituitary from D_2R knockout mice (Figure 1, $D_2R^{-/-}$). Similar results were obtained when $[^3H]$-spiperone was used (not shown). The lack of binding of these compounds in D_2R-null pituitaries indicates that neither D_3R nor D_4R can compensate for the absence of D_2R, and we also question their reported presence in WT pituitaries.

Pituitary Hyperplasia in D2R-Null Mice

A key question concerns the role of DA acting through the D_2R on the normal differentiation program of the pituitary gland. Thus, to characterize the pituitary phenotype of D_2R knockouts, we first performed histological analyses of pituitary sections from $D_2R^{-/-}$ and WT animals. The pituitaries of 4-month-old animals ($n > 10$) of both sexes and genotypes were analyzed. Cryostat and paraffin sections were prepared and stained with hematoxylin-eosin. Sections obtained from D_2R-null 4-month-old females and males compared with WT littermates are presented in Figure 2A. Strikingly, it is evident that the overall size of the pituitary is augmented in both male and female $D_2R^{-/-}$ mice, although this enlargement is less pronounced in males (Figure 2A). Diamidinophenolindole (DAPI) staining of pituitary cell nuclei in these sections revealed a hyperplasia of the IL (Figure 2B, top panel). The surface of the intermediate lobe showed a 40% increase in $D_2R^{-/-}$ mice (WT = 0.28 ± 0.03 mm^2; $D_2R^{-/-}$ = 0.4 ± 0.04 mm^2; $n = 40$ sections from six pituitaries of each genotype), which resulted in a corresponding increase in cell number but not in cell density. Conversely, in the AL, both cell number and density were similarly increased, as demonstrated by hematoxylin-eosin staining (Figure 2B, bottom panel). This indicates that lack of dopaminergic control leads to pituitary cell proliferation.
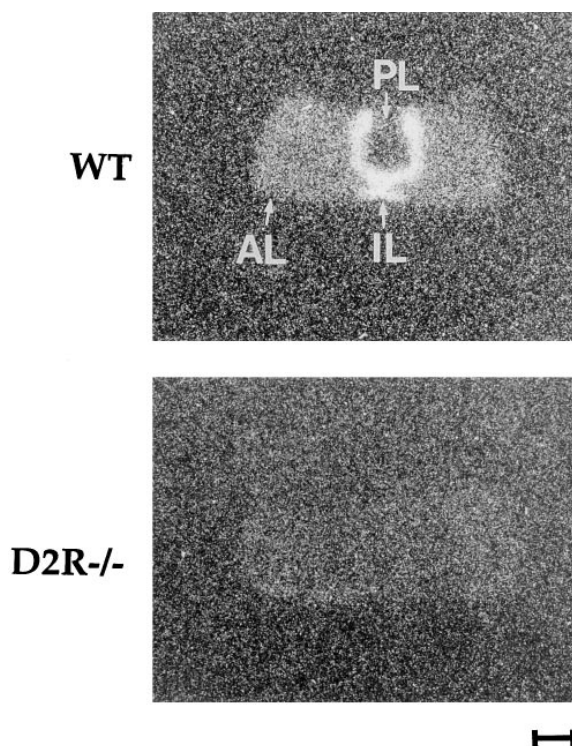

Figure 1. Ligand Binding Autoradiography of WT and $D_2R^{-/-}$ Pituitaries

Ligand binding experiments were performed with $[^{125}I]$-iodosulpride. The AL and IL of the WT pituitary are strongly labeled, whereas labeling is completely absent in the $D_2R^{-/-}$ pituitary. Results are representative of three independent experiments. AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe. Scale bar, 500 μm.

Functional Consequences of D_2R Absence on Pituitary Hormones

The enlargement of the AL and IL correlates with the location of the two cell types that normally express D_2Rs: lactotrophs and melanotrophs (Cote et al., 1986; Elsholtz et al., 1991; Ben-Jonathan, 1994). Thus, we analyzed whether this abnormality might influence other parameters such as the expression of pituitary hormones. Total RNA extracted from pituitaries of 4-month-old $D_2R^{-/-}$ mice and WT littermates was analyzed by Northern blot using specific probes for the different pituitary hormones (Figure 3). A threefold increase of PRL mRNA expression and a three- to fourfold increase of POMC gene expression were found in pituitaries from D_2R-null mice, compared with WT littermates. The increase of POMC expression is restricted to melanotrophs, as revealed by RNAse protection experiments, in which the AL was separated from the IL (data not shown). This is in agreement with the restriction of D_2R expression to the melanotrophs and not the corticotrophs, the other pituitary cell type which expresses POMC.

The level of expression of the hormones produced by the other AL cell types was evaluated by using probes specific for thyrotrophs (β-TSH, αGSU), somatotrophs (GH) and gonadotrophs (β-FSH, β-LH, αGSU). The result of this analysis shows a 20% decrease in the expression of GH, while no difference in β-TSH expression (Figure
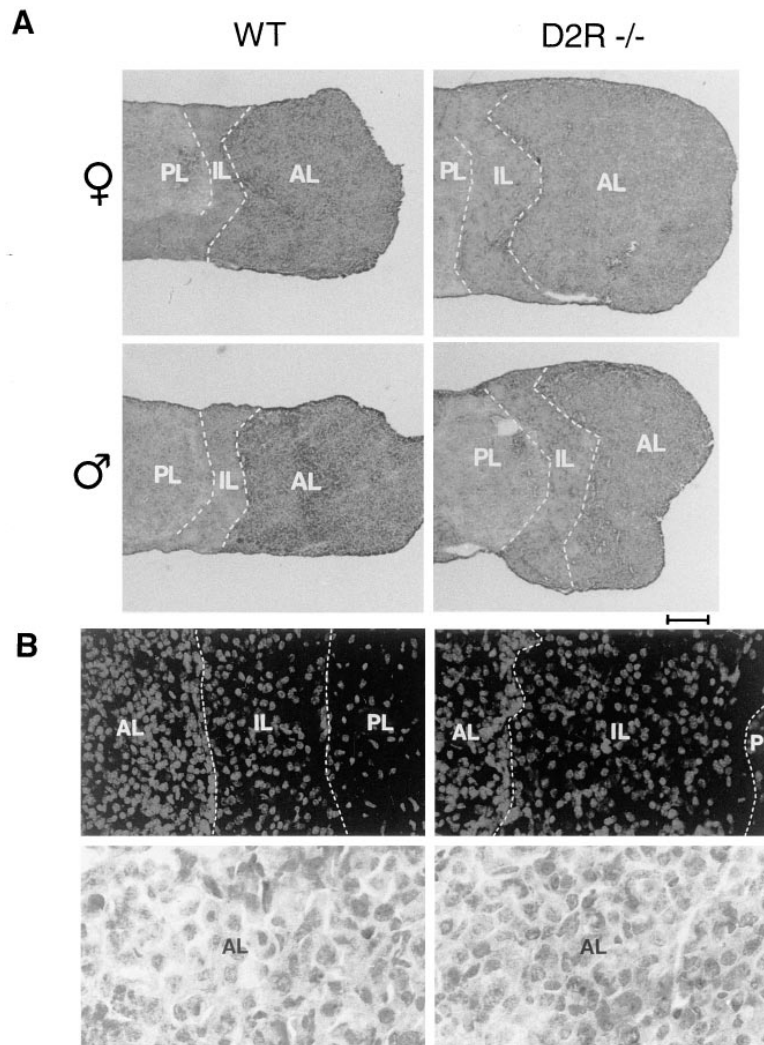
Figure 2. Histological Analysis of the Pituitary Gland of WT and $D_2R^{-/-}$ Mice

(A) Hematoxylin-eosin staining of pituitary cryostat sections from 4-month-old WT and $D_2R^{-/-}$ females and males, as indicated. Scale bar, 200 μm.

(B) The top row shows DAPI staining of the IL from WT and $D_2R^{-/-}$ female pituitaries. Dotted lines indicate the boundaries between the different lobes. The bottom row shows hematoxylin-eosin coloration of the AL. Original magnification, 200×. AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe.

3) was observed. Considering the increased size of the AL, these results are in agreement with a reduction of GH-positive cells and an increase in TSH-positive cells. We also confirmed that the reduction in GH is not dependent upon an altered expression of GHRH, since no difference in its levels was observed in hypothalamic RNA from WT and D_2R-null mice (data not shown). Interestingly, β-LH and β-FSH expression was reduced by 20% (Figure 3). The reduction of gonadotrophin mRNAs is probably dependent on the increased PRL level, since PRL exerts a negative control on the hypothalamic release of gonadotrophin-releasing hormone (Li and Pelletier, 1992). In agreement, Northern analysis of αGSU, the common subunit of glycoproteic hormones expressed specifically by gonadotrophs and thyrotrophs (Pierce and Parsons, 1981; Akerblom et al., 1990; Kendall et al., 1995), showed no differences in the expression of this gene (Figure 3). Thus, D_2R absence leads to alterations of AL hormone expression.

The Loss of D_2Rs Affects the Thyro-Somato-Mammotroph Cell Lineage

In addition to increased PRL expression, loss of D_2R in the AL also results in a decrease of GH expression. This indicates that in the adult, DA, through D_2Rs, has an important role in maintaining lactotrophs and somatotrophs, which share common developmental origins with thyrotrophs. The availability of genetic mutants, as well as cell ablation studies (Behringer et al., 1988; Borrelli et al., 1989), enabled the development of the thyro-somato-mammotroph lineage to be elucidated. The analyses of these model systems have demonstrated that these cells derive from a common precursor, and that expression of Pit-1 (Li et al., 1990; Rhodes et al., 1994) and Prop-1 (Sornson et al., 1996) is an essential requirement for fate cell commitment. Later in development, GHRH is required for their proliferation (Mayo, 1992; Lin et al., 1992; Godfrey et al., 1993).

Estrogens have been shown to stimulate lactotroph proliferation in the adult (Lieberman et al., 1983), whereas DA has been only indirectly implicated in the control of the proliferation of this lineage. Indeed, treatment with dopaminergic agonists helps the regression of prolactinomas, PRL-producing human pituitary tumors (Bansal et al., 1981). However, alone, these observations do not clearly define the role of DA in the normal physiology of lactotrophs, nor the molecular mechanism of its action.
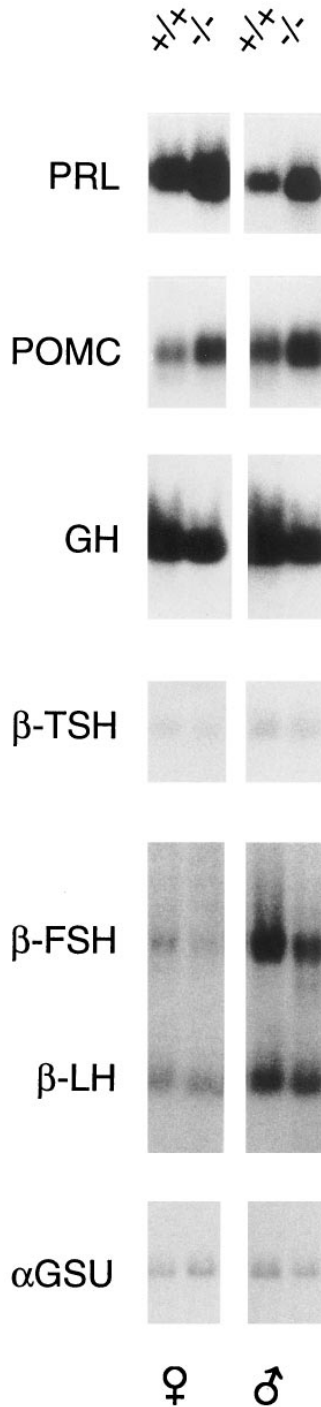
Figure 3. Pituitary Hormone Expression in WT and $D2R^{-/-}$ Mice

Northern blot analysis of PRL, POMC, GH, β-TSH, β-FSH, β-LH, and αGSU pituitary mRNAs from WT (+/+) and D₂R mutant (−/−) mice. The amounts of total RNA per sample were as follows: PRL, 0.5 μg (females) and 2 μg (males); GH and αGSU, 1 μg; POMC, 3 μg; β-TSH, β-FSH, and β-LH, 5 μg. Results are representative of three independent experiments.

We therefore focused our attention on the study of this lineage in D₂R-null animals. In situ hybridization experiments were performed to confirm whether the observed change in GH and PRL mRNA levels was due to

shifts in the proportion of their respective hormone-producing cells. These analyses (Figure 4) illustrated that the PRL mRNA elevation is at least in part dependent on an increase in the proportion of lactotrophs in D₂R-null mice. A robust PRL expression is visible in the tissue from D₂R-null mice (Figure 4). An increased number of lactotrophs is also evident, with PRL positive cells appearing smaller and more compact (Figure 4).

Thyrotrophs constitute a very scarce population (2%–5%) of AL pituitary cells (Gage et al., 1996). Interestingly, a twofold increase in the number of thyrotrophs was observed in the pituitary of D₂R-null mice (40 ± 12 cells/section; WT = 22 ± 9 cells/section; n = 24 sections from four pituitaries of each genotype; $P < 0.05$, Student's t-test) (Figure 4, β-TSH). This was evaluated by the counting of cells containing autoradiographic grains in serial sections of the gland. These data indicate that the expansion of the AL might also involve these cells. In spite of the increased cell number, no significant increase of β-TSH plasma levels was present in D₂R-null mice (n = 8 males and 9 females) as compared with WT (n = 10 males and 9 females). On the contrary, we estimated a 25% decrease in the number of GH-expressing cells in these mice as compared with WT littermates (WT = 214 ± 45 cells/0.1 mm²; D₂R-null = 162 ± 30 cells/0.1 mm²; n = 200 counting fields from four pituitaries of each genotype; $P < 0.001$, Student's t-test) (Figure 4, GH). In agreement with this finding, GH plasma levels were decreased by 25% and 35% in D₂R-null males and females, respectively (n = 10–12 animals per experimental group).

The number of the remaining AL cells, gonadotrophs and corticotrophs, appeared unaltered (data not shown). Therefore, the major effect in the AL caused by the loss of D₂Rs concerns the cell proportion within the thyro-somato-mammotroph lineage. Thus, DA controls lactotroph proliferation, and in its absence more somatotrophs are converted into lactotrophs. This effect could be due to an imbalanced estrogen stimulation of lactotroph proliferation in D₂R-null mice.

Hyperprolactinemia in the Absence of Dopaminergic Control

Consistent with the massive increase of lactotrophs, quantitation of serum PRL revealed very high levels of this hormone in D₂R-null mice. Adult female PRL plasma levels increased from 24.2 ± 5.4 ng/ml serum in the WT (n = 10) to 264 ± 35.2 ng/ml in the mutant (n = 10), whereas in adult males, levels increased from 7.7 ± 0.8 ng/ml in the WT (n = 10) to 41.1 ± 7.2 ng/ml in the mutants (n = 10). D₂R-null homozygotes showed a 10.9- and 5.3-fold higher PRL serum content in females and males, respectively. Importantly, the elevation of PRL in the serum is much greater than what would have been predicted from the increase in the levels of mRNA and lactotroph number alone (Figures 3 and 4).

Neuroregulation of PRL secretion is multifactorial. Although the hypothalamic control is mainly negative and exerted by DA, positive signals are also needed to induce the acute secretion of PRL and include the thyrotropin-releasing hormone (TRH) (Jacobs et al., 1971) and vasoactive intestinal peptide (VIP) (Abe et al., 1985). Consequently, we analyzed the expression of these two
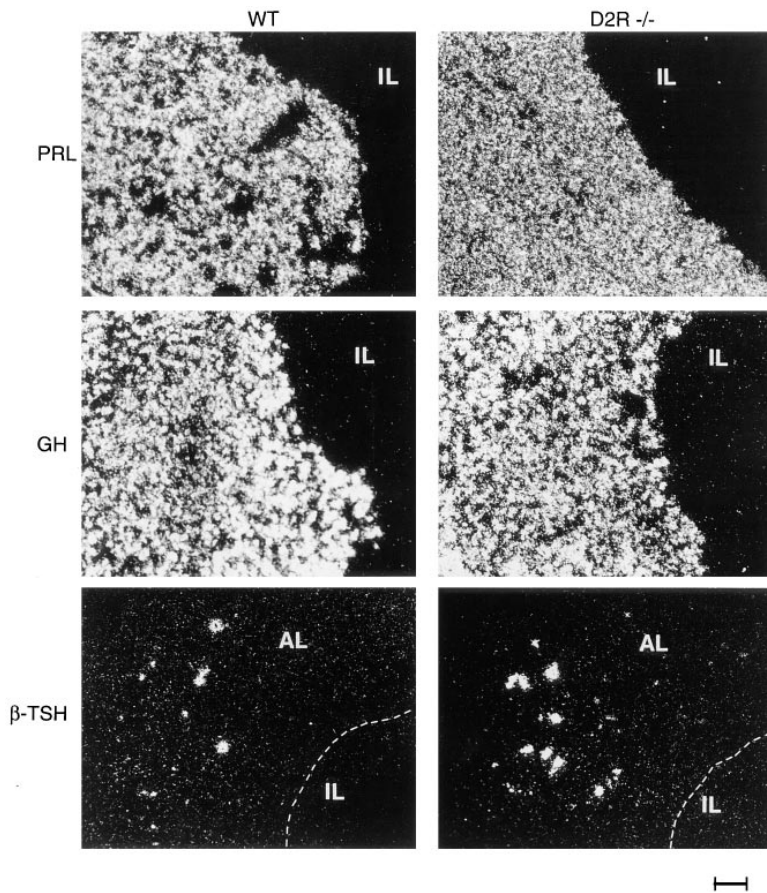
Figure 4. In Situ Analysis of PRL, GH, and β-TSH mRNA Expression in the Pituitary Gland of WT and $D_2R^{-/-}$ Female Mice

Serial sections from pituitaries of 4-month-old WT and $D_2R^{-/-}$ females were hybridized with mouse PRL, GH and β-TSH probes. Results are representative of four different experiments. Dotted lines indicate the border between AL and IL. AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe. Scale bar, 75 μm.

positive acting factors in the D_2R-null mice. Interestingly, in situ hybridization analysis of hypothalamic tissue from D_2R-null and WT littermates showed similar levels of expression of the TRH and VIP genes in both genotypes (Figure 5). This indicates that lack of D_2R does not seem
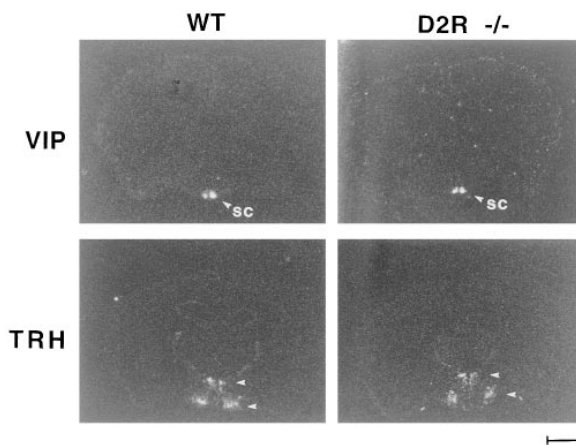

Figure 5. In Situ Analysis of VIP and TRH mRNA Expression in the Hypothalamus of WT and $D_2R^{-/-}$ Mice

Serial sections from the brain of WT and $D_2R^{-/-}$ females were hybridized with mouse VIP and TRH probes. Similar results were obtained in three independent experiments. SC, suprachiasmatic nuclei. The arrowheads in the TRH pictures indicate the periventricular hypothalamic region. Scale bar, 125 μm.

to affect the expression of hypothalamic hormones that regulate PRL secretion. Thus, the observed increase of serum PRL seems to be directly linked to the absence of dopaminergic control on the secretion of these hormones. Therefore, D_2R appears to exert a stronger inhibitory control over prolactin release than on RNA transcription/stability. One possibility is that, in the absence of D_2Rs, the tonic PRL release from lactotrophs is lost, and the hormone is constitutively secreted in D_2R-null mice. Accordingly, in spite of the increased lactotroph population in D_2R-null mice, immunoblot analysis using anti-PRL antibodies revealed a 50% decrease of PRL in the pituitary gland of D_2R-null mice (not shown).

Hypoestrogenism in D_2R Deficient Mice
Extensive evidence clearly demonstrates stimulation of lactotroph cell proliferation by estrogens. Moreover, estrogens have been shown to play a permissive role in directing the somato-mammotroph lineage toward lactotroph differentiation (Boockfor et al., 1986). Thus, in the absence of the negative control exerted by D_2R on lactotrophs, estrogens might stimulate the proliferation of these cells. However, it is known that high PRL levels lead to hypoestrogenism, which in turn results in infertility in humans (Wang et al., 1980; Dorrington and Gore-Langton, 1981). Interestingly, D_2R-null animals have a reduced fertility (Baik et al., 1995), indicating perturbations of the pituitary–gonadal axis.

To gain further insight into the mechanisms underlying
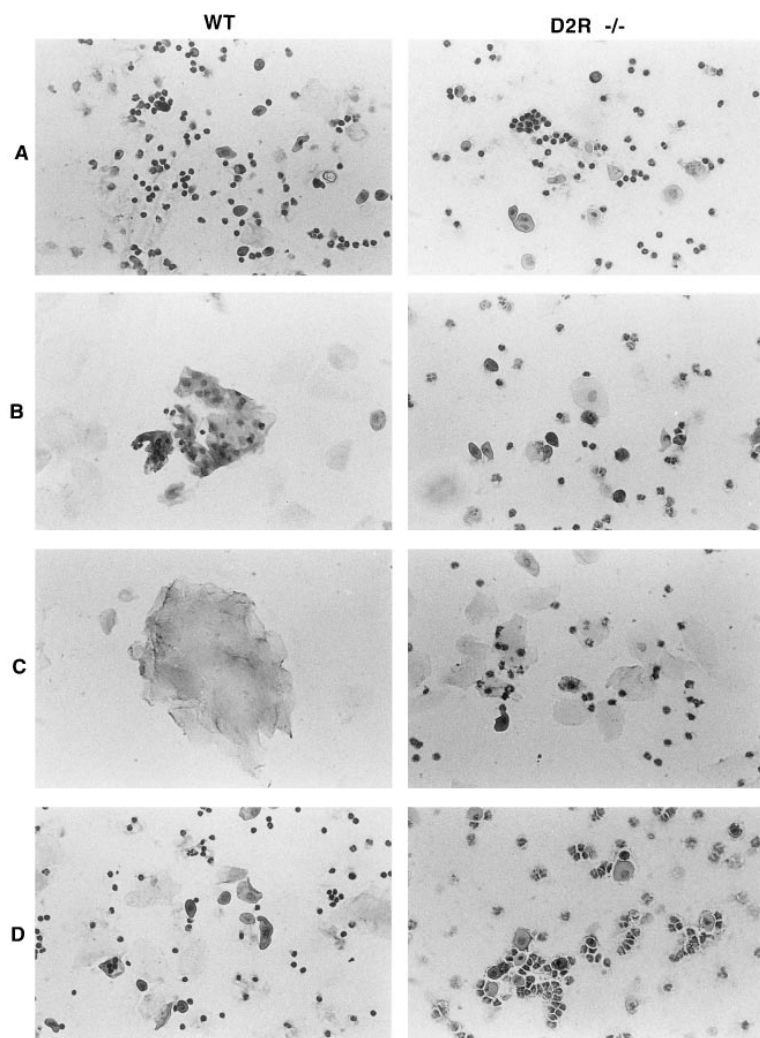
WT   D2R -/-

Figure 6. Analysis of the Estrous Cycle in WT and $D_2R^{-/-}$ Female Mice

Vaginal smears from a WT and a $D_2R^{-/-}$ mouse stained by hematoxylin-eosin. In WT females, the estrous cycle has a characteristic periodicity, with an estrus every 4 days. Diestrus (WT in [A]) is characterized by the prevalence of lymphocytes (small nucleated cells) in the vaginal fluid. During proestrus (WT in [B]), the vaginal fluid begins to contain nucleated epithelial cells, due to the keratinization of the upper layer of the vaginal epithelium. In the estrous stage (WT in [C]), cells in the vaginal fluid are only keratinocytes. During metaestrus (WT in [D]), the keratinization of the upper layers of the vaginal epithelium decreases, and both keratinocytes and lymphocytes can be observed. Conversely, all vaginal smears collected from $D_2R^{-/-}$ mice reveal the continuous presence of lymphocytes ($D_2R^{-/-}$, A–D), indicating a complete alteration of the estrous cycle. Original magnification, 40×.

lactotroph proliferation, we assessed the estrogen levels in D_2R mutant mice. Comparative analyses of the estrous cycle of D_2R-null females (n = 5) and WT mice (n = 5) were performed. Interestingly, while a rhythmic periodicity with a recognizable estrus is evident every 4 days in WT females, the estrous cycle of D_2R mutants was completely altered (Figure 6). Indeed, estrus was observed only once in a month in D_2R-null females. These data strongly indicate that high levels of PRL perturbate gonadal function in female mutants. In contrast, D_2R-null males display normal spermatogenesis.

Next, we analyzed estrogen levels in D_2R-null and WT animals. Since the mutant females have a sporadic estrus, it was impossible to collect blood at definite cycle stages. We thus pooled blood samples from 13 individuals of each group (housed in different cages) to estimate the average estrogen levels. In agreement with the estrous cycle data, D_2R-null females have a 70% reduction of estrogen serum content as compared to WT females. Estrogens were also reduced by 20% in males. Interestingly, estrogen levels in D_2R-null mice were very similar in the two genders (females, 8 ng/l; males, 9 ng/l). Thus, in contrast to the WT mice, PRL transcription is presumably equally stimulated by estrogens in these mice. Nevertheless, D_2R-null females still

presented a striking difference in the PRL plasma levels compared with mutant males (females, 264 ± 35.2 ng/ml; males, 41.1 ± 7.2 ng/ml). While WT females have a PRL concentration three times higher than males, it is six times higher in the knockouts. Therefore, the physiological causes for the differences in the PRL levels between males and females are different in WT and D_2R-null mice. In WT females, high PRL plasma levels are due to the synergistic contribution of high estrogens and a larger number of lactotrophs. The increase of PRL in D_2R-null females versus males must be solely linked to the enrichment for lactotrophs.

Is Prolactin a Prolactin-Releasing Factor?

A key question arises from these results: what determines the increase in the lactotroph population and why is this effect more prominent in females? As demonstrated, neither estrogens or hypothalamic factors seem to be implicated in the D_2R-null mice. Thus, the only likely explanation is that PRL itself stimulates lactotroph proliferation. Interestingly, this effect appears to be slow, continuous, and dependent on threshold PRL levels. Male knockouts do have hyperplastic pituitaries, but not as prominent as in age-matched females (Figure
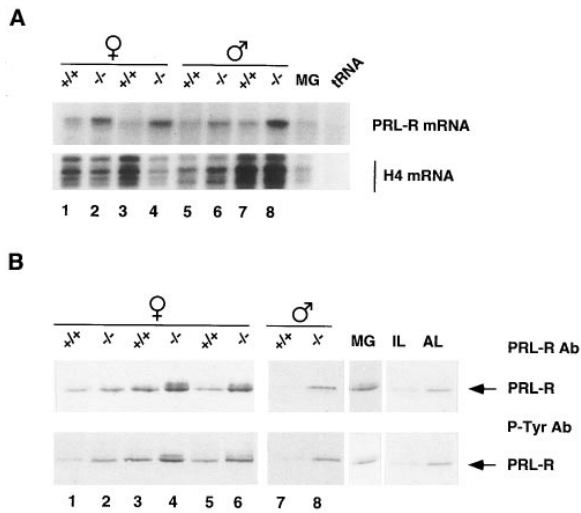
A

B

Figure 7. Expression and Activation of PRL-R in the Pituitary Gland of $D_2R^{-/-}$ Mice

(A) RNAse protection analysis of PRL-R mRNA in the pituitary gland of WT and $D_2R^{-/-}$ mice. Lanes 1 and 2, 4-month-old females. Lanes 3 and 4, 1-year-old females. Lanes 5 and 6, 4-month-old males. Lanes 7 and 8, 1-year-old males. MG, WT mouse mammary gland. H4 histone was used to normalize the quantity of RNA loaded. Results are representative of three independent experiments.

(B) The top row shows immunoprecipitation of pituitary extracts from WT and $D_2R^{-/-}$ mice with an anti-JAK2 antibody. Immunoprecipitates were revealed with the anti-PRL-R antibody U5. Lanes 1 and 2, 4-month-old females. Lanes 3 and 4, 8-month-old females. Lane 5 and 6, 1-year-old females. Lanes 7 and 8, 4-month-old males. MG, mouse mammary gland. AL and IL indicate extracts from isolated anterior and intermediate lobes.

The bottom row shows immunoprecipitation of pituitary extracts from WT and $D_2R^{-/-}$ mice with the JAK2 antibodies. Immunoprecipitates were revealed with an anti-phosphotyrosine antibody (P-Tyr). Samples were loaded in the same order as in the top panel. Results are representative of three independent experiments.

2A), while PRL plasma levels are sixfold lower in male than in female knockouts.

These results strongly support the hypothesis that the slow developing hyperplasia in D_2R-null females is due to an autocrine effect of PRL on lactotroph cells. However, a prerequisite for this hypothesis is that functional prolactin receptors (PRL-R) (Boutin et al., 1988) should be found in the mouse pituitary. To date, the expression of PRL-R has been reported only in the rat anterior pituitary (Morel et al., 1994). Thus, we first performed RNAse protection analyses to detect the expression of PRL-R mRNA in the pituitaries of WT and $D_2R^{-/-}$ mice. A protected fragment corresponding to the PRL-R was observed in the pituitaries of WT mice. A fourfold increase of PRL-R mRNA was found in 4-month-old D_2R-null mice, whereas a 10-fold increase was observed in older mutants (Figure 7A). Western blot analyses using the anti-PRL-R antibodies, performed after immunoprecipitation of pituitary extracts from female and male WT and D_2R-null mice at different ages, confirmed these data. A band corresponding to the PRL-R is present in the pituitaries of WT and D_2R-null animals (Figure 7B). The identity of the immunoreactive band obtained with the PRL-R antibody was confirmed by the use of extracts from mouse mammary gland, one of the major PRL-R expression sites (Figure 7B) (Jahn et al., 1991). Interestingly, the intensity of the band in male mouse pituitaries is weaker than in females (Figure 7B, compare lanes 7 and 1 and lanes 8 and 2). The comparison of pituitary extracts from WT and D_2R-null mice shows an increase of PRL-R in knockout animals. This difference becomes more pronounced as the animals become older (Figure 7B). Dissection of the IL from the AL shows that the PRL-R is also expressed in the IL (Figure 7B). Thus, the hyperplasia correlates with a progressive induction of PRL-R levels in the pituitary gland of the D_2R-null mice.

Interaction of the PRL-R with PRL is known to lead to its phosphorylation by the activity of Janus kinases (JAK) (Rui et al., 1994; Schindler and Darnell, 1995; Lebrun et al., 1995; Gao et al., 1996). Thus, to show that the pituitary PRL-R is functional, we probed the immunoprecipitates with an anti-phosphotyrosine antibody. The banding pattern we obtained is indicative of active receptors since the intensity of the bands obtained with the anti-phosphotyrosine antibody corresponds to those detected with the PRL-R antibody (Figure 7B).

We conclude that the pituitary hyperplasia observed in D_2R-null mice is dependent on the aberrant PRL levels resulting from the lack of dopaminergic inhibition. Thus, we propose that PRL may play an autocrine role in pituitary cell proliferation.

D_2R Deficient Females Develop Pituitary Tumors by Eight Months of Age

The increased size of the pituitary gland observed in 4-month-old animals prompted us to analyze older animals, to study whether this effect may be amplified with time. Strikingly, the dissection of ten 8- to 14-month-old D_2R-null females revealed the presence of anterior pituitary tumors in all of the animals analyzed. The tumoral growth was independent of whether or not these females had been pregnant or of the number of pregnancies they had had during their life. The pituitary gland of knockout males, although enlarged, never showed a similar hyperplasia at this age. However, in 19-month-old D_2R-null males, a phenotype similar to the one exhibited by the 8- to 14-month-old D_2R-null females was observed. In contrast, no signs of hyperplasia were encountered in the animals from the control group. The histological appearance of the AL of one of these pituitaries is illustrated in Figure 8. Cryostat sections stained by hematoxylin-eosin showed that the tumor specifically affects the AL, with nodular areas and large blood vessel infiltration, a typical feature of pituitary adenoma. The IL is also hyperplastic but still contained and histologically well defined. Furthermore, the characteristic three-lobed structure of the pituitaries is still recognizable.

In order to characterize the cell types present in these tumors, we performed in situ hybridization of serial pituitary sections from 14-month-old D_2R-null females using probes corresponding to the genes encoding the different hormones. The hyperplastic anterior lobe still contains gonadotrophs expressing β-FSH and β-LH and POMC-positive corticotrophs. The number of these cells
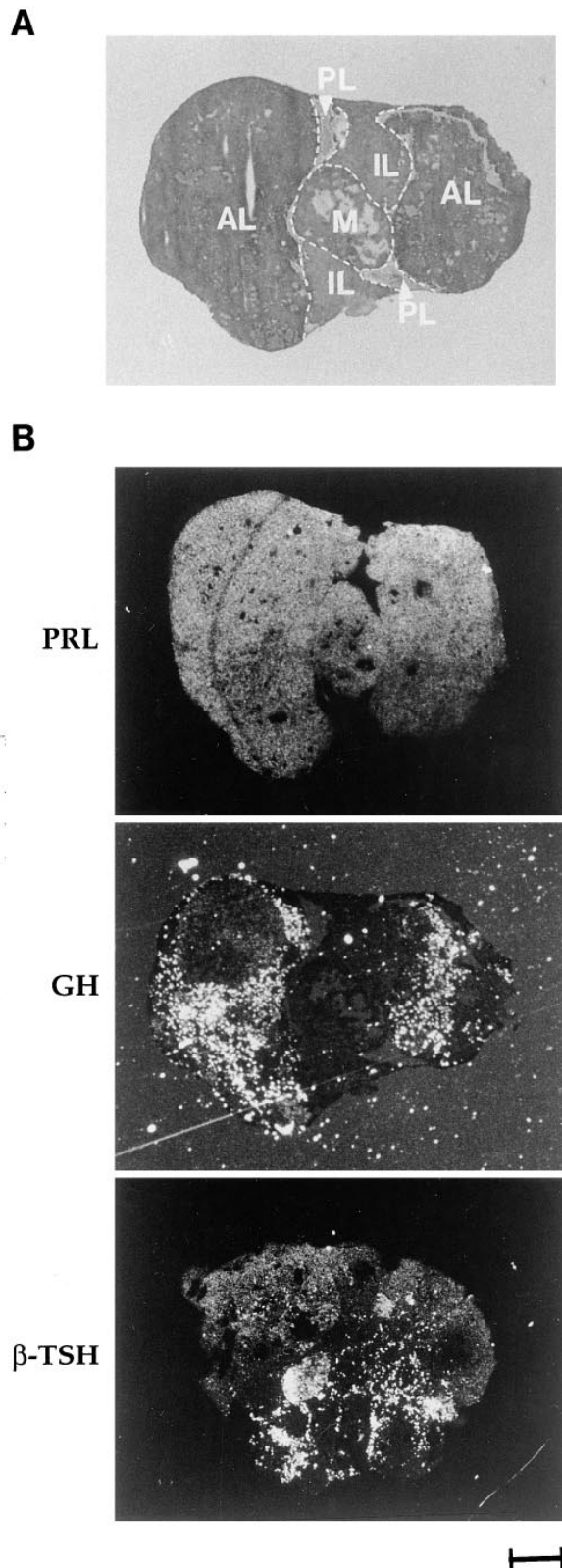
A

B

PRL

GH

β-TSH

is not modified with respect to WT mice, and they appear sparse in the overgrowing tissue (data not shown). In sharp contrast, the thyro-somato-lactotroph lineage is profoundly affected in these pituitaries. We observed a robust PRL labeling in sections. Indeed, proliferation of PRL-producing cells is the basis of the tumor, since there are no regions of the anterior lobes devoid of PRL expression (Figure 8). Surprisingly, the number of GH-expressing cells, which was already reduced in younger animals (Figure 4), is further decreased (Figure 8). In contrast, an increase of β-TSH positive cells is observed (Figure 8). It should be pointed out that thyrotrophs represent only 2%–5% of a normal AL cell population (Gage et al., 1996). These results indicate that the lack of DA D_2Rs in lactotrophs results in a continuous proliferation of these cells throughout the animal's life. Lactotroph proliferation causes a dramatic decrease in the number of cells expressing GH. As previously suggested, the somato-mammotroph lineage might follow an alternative differentiation route, ultimately leading to an over-production of lactotrophs. The increase of thyrotrophs could reflect a compensatory mechanism, in which the continuous transformation of somatotrophs into lacto-trophs activates the proliferation of this lineage from a common stem cell (Gage et al., 1996) still present in the adult (Borrelli et al., 1989). Transcription factor Pit-1/ GHF1 is required for the transcription of the hormones produced by these cells and for their commitment (Lin et al., 1992; Andersen and Rosenfeld, 1994). In vitro studies have shown that the Pit-1 promoter is under a negative dopaminergic control elicited by the D_2Rs (Elsholtz et al., 1991). We thus analyzed whether the observed phenotype might involve an upregulation of Pit-1 in the thyro-somato-mammotrophs of D_2R-null mice. The analysis was conducted on pituitaries of 4- and 14-month-old WT and mutant animals. Pit-1 expression was confined to the AL in both group of animals, and the level of expression per cell did not differ in the mutant animals as compared to WT littermates (data not shown).

Discussion

The development of the pituitary gland is under the control of various factors involved in the establishment, proliferation, and maintainance of cell type (Voss and Rosenfeld, 1992; Borrelli, 1994). In this paper, we reveal a central involvement of DA in the control of these functions. Absence of D_2R leads to an increased expression of the hormones produced by the lactotrophs and mela-notrophs, indicating that, in both cases, DA exerts an inhibitory control over the expression of these genes. Interestingly, the histology of the pituitary of young D_2R-null animals shows a clear enlargement of the inter-mediate and anterior lobes in both sexes. The expansion of the lobes is mirrored by a substantial increase of PRL and POMC gene expression. We show that this increase

Figure 8. Abnormal Growth and Hormone Expression in the Pituitary of a 14-Month-Old $D_2R^{-/-}$ Female

(A) Hematoxylin-eosin staining of a cryostat section. The figure shows the typical aspect of tumoral pituitaries from aged $D_2R^{-/-}$

females. AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe; M, tumoral mass.

(B) PRL, GH, and β-TSH in situ hybridization of serial sections from the same pituitary as in (A). Scale bar, 500 μm.

is mainly due to a striking proliferation of the cells that express these hormones, the lactotrophs and the melanotrophs. Both cell types normally express D_2Rs. Thus, DA through D_2R controls the rate of proliferation of pituitary cells. Importantly, mutant mice lacking the DA transporter (Giros et al., 1996) present an increased dopaminergic tone that causes dwarfism and pituitary hypoplasia (Bossé et al., 1997 [this issue of Neuron]). In these mice, the cell number of both the melanotrophs and lactotrophs is greatly reduced, further suggesting that the principal role of DA in pituitary physiology is at the level of cell proliferation.

We have observed that the estrous cycle of D_2R-null females looses its strict periodicity (Figure 6) and occurs only once a month. These results establish a link with the observations that hyperprolactinemia leads to hypoestrogenism in humans (Frohman, 1987). It is known that estrogens play a critical role in lactotroph proliferation as well as in the stimulation of prolactin synthesis (Day et al., 1990), and it has been proposed that estrogen might be the factor required for lactotroph growth (Slabaugh et al., 1981; Carbajo-Perez and Watanabe, 1990; Voss and Rosenfeld, 1992; Gage et al., 1996). In the present study, we show that DA is implicated in the regulation of lactotroph proliferation, since the absence of D_2Rs leads to pituitary hyperplasia and tumors. This process, apparent by the first months of age, becomes extremely pronounced in 8- to 10-month-old female D_2R-null mice, indicating the presence of a constant proliferative stimulus on lactotrophs. This is not dependent on alterations of hypothalamic releasing factors such as TRH and VIP as demonstrated by our results. Estrogen stimulation of lactotroph growth also cannot account for this effect, since D_2R-null females have estrogen plasma levels reduced by 70% with respect to WT mice. Interestingly, D_2R-null males and females have similar estrogen levels, although sexual differences are present at the level of PRL plasma concentration. These differences can only be explained by the presence of a much larger number of lactotrophs in females versus male mice. Our data demonstrate that lactotroph hyperplasia is dictated by threshold levels of PRL. Indeed, both the hyperplasia and the tumor formation occur later in males than in females, due to lower PRL levels in males. These results lead us to propose that PRL itself plays an autocrine role in pituitary cell proliferation.

D_2R-null females present alterations of the thyrosomato-mammotroph lineage. The number of somatotrophs is reduced while the thyrotrophs are increased in the hyperplastic pituitaries. It is conceivable that in these animals more somatotrophs are transformed into lactotrophs. This process is likely to be triggered as soon as PRL levels increase above a certain threshold, being evident in 4-month-old mice and then increasing in older animals. This effect also leads to an increase in thyrotrophs. These cells probably derive from precursor stem cells still present in the adult (Borrelli et al., 1989). These results indicate that DA is of fundamental importance in the maintainance of the adult pituitary phenotype by repressing synthesis and release of PRL, and they also reveal a pivotal function for prolactin in pituitary cell development.

Previously, PRL-R expression was documented in the rat anterior pituitary (Morel et al., 1994). Interestingly, a gradation of PRL-R density has been shown between the different AL cell types, with the somatotrophs showing the highest expression (Morel et al., 1994). We demonstrate that PRL-Rs are also present in the mouse pituitary, and we extend this observation to the melanotrophs in the IL (Figure 7). The PRL-R is present and activated since it is phosphorylated (Figure 7). The activation of the PRL-R transduction pathway is known to lead to altered cell proliferation (Mershon et al., 1995). Interestingly, lactotrophs are the last cells to differentiate during pituitary development. Since PRL is first detected around postnatal day 5 (Frawley and Miller, 1989), this suggests that the proliferative function of this hormone might operate following the complete development of the other cell types. As mentioned above, the PRL-Rs are highly concentrated on somatotrophs, which are able to proliferate also in the adult; conversely, lactotrophs are postmitotic cells, as shown from ablation studies (Borrelli et al., 1989). This raises the intriguing possibility that PRL might mediate lactotroph proliferation by activating PRL-Rs present on somatotrophs, causing them to differentiate into lactotrophs. The key regulator of such function is the D_2R that controls PRL expression and release. In the absence of D_2Rs, PRL is continuously secreted, generating the hyperplasia. In keeping with our findings, there is evidence that one of the most common pituitary tumors, the prolactinoma, originates from a lack of dopaminergic inhibition (Wood et al., 1991). These tumors normally regress following D_2R agonist treatment, and in the most malignant cases these tissues do not respond to this treatment, suggesting a total loss of dopaminergic control.

Plasticity in lactotroph growth can be influenced by different factors. It has been shown that overexpression of nerve growth factor (NGF) under the control of the prolactin promoter in transgenic mice results in high PRL synthesis and lactotroph hyperplasia (Borrelli et al., 1992), a pituitary phenotype similar to the one described in this paper. Interestingly, the presence of NGF receptors on lactotrophs has been reported recently, and the trophic effect of NGF on these cells has been demonstrated (Missale et al., 1996a, 1996b). The activation of the signal transduction pathways by PRL and NGF, although done through different signal transduction mechanisms (Schindler and Darnell, 1995; Segal and Greenberg, 1996), lead to a similar effect on cell proliferation. It is tempting to speculate that during normal pituitary cell differentiation NGF, via an autocrine mechanism or through the stimulation of PRL synthesis, could be responsible for lactotroph growth. In this paper, we show that only the lactotrophs and melanotrophs are affected by the elevation of PRL, despite the presence of PRL-R on all cell types of the anterior pituitary. Melanotrophs and lactotrophs are the only two cell types expressing D_2Rs and consequently are the only ones to have lost the hypothalamic inhibitory control of growth as a result of D_2R ablation.

Our results illustrate the critical role of D_2R in the pituitary and on the neuroendocrine control of physiological functions. They also assign a new and important role to PRL in pituitary cell proliferation. We propose that receptor-mediated activation of the tyrosine kinase

pathway might play a key role in the control of pituitary cell proliferation and maintenance.

Experimental Procedures

Mice

D_2R mutants (Baik et al., 1995) had a mixed 129SV×C57Bl/6 genetic background, with a 75% contribution of C57Bl/6. Animals were housed in a 12 hr light/dark cycle with food and water available *ad libitum*. Wild-type and knockout littermates obtained by crossing of heterozygotes were used in all experiments.

Ligand Binding

Four-month-old wild-type (n = 3) and mutant (n = 3) mice were sacrificed after anesthesia (Rompun, Bayer). Pituitary cryostat sections (10 μm) were mounted on gelatine-coated slides and stored at −80°C. Sections were brought to room temperature, washed in HEPES buffer (50 mM Hepes [pH 7.5], 1 mM EDTA, and 0.1% BSA) for 5 min and incubated for 1 hr at room temperature with 0.3 nM [^{125}I]-iodosulpride (2000 Ci/mmol; Amersham, UK) in Tris-buffered salt solution (50 mM Tris [pH 7.5], 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 nM ascorbic acid, and 10 μM hydroxyquinoline). Nonspecific binding was determined with 5 μM cold sulpiride. After incubation, sections were washed twice for 2 min in ice-cold Tris-buffered salt solution and dipped in cold distilled water. Sections were dried and exposed for 24–72 hr with autoradiographic films.

Histological Analyses

Cryostat and paraffin sections of pituitary glands from 12 WT and 14 knockout mice of both sexes were used. For paraffin sectioning, pituitaries were fixed in Bouin's fixative, embedded in paraffin, and cut with a microtome (10 μm). Sections were stained by hematoxylin-eosin or by DAPI staining. The surface (± SD) of the IL of WT and D_2R-null mice was estimated by analyzing sections, all of which contained a well defined IL, using the NIH Image 1.59 software. A similar number of cells/unit (1 unit = 0.1 mm^2) was evaluated by counting DAPI stained nuclei from 40 sections from six animals of each genotype. Numbers are estimates of profile densities and, as such, have the caveat of being offset by changes in the volume of the pituitaries.

For the analysis of the estrous cycle, vaginal smears from five WT and five $D_2R^{-/-}$ adult mice were collected every day for a 40 day period. Smears were streaked on slides and stained by hematoxylin-eosin.

RNA Analysis

GH, PRL, β-TSH, POMC, and PRL-R cDNAs were obtained by RT-PCR from mouse pituitary RNA using oligos based on published sequences (GenBank accession numbers: X02891, X02892, J00644, J00610, and L13593). TRH and VIP probes were amplified from genomic DNA (GenBank accession numbers: X59387 and X74297). β-LH, αGSU and β-FSH cDNAs were a generous gift from Dr. P. Mellon.

Northern Blot Analysis

Total RNAs were prepared from pituitaries by the LiCl method (Sambrook et al., 1989). Three animals of both sexes and genotypes were tested in three separate experiments. Equal amounts of RNA from WT and $D_2R^{-/-}$ mice were run on formaldehyde/agarose gels in 10 mM sodium phosphate buffer and transferred to Hybond N+ (Amersham, UK). β-actin was used as an internal standard for RNA quantitation. Quantification was performed with a Fuji Bio-Imaging Analyzer BAS 2000.

RNAse Protection

RNAse protections were performed as described (Sambrook et al., 1989). Three animals of both sexes and genotypes were used in three independent experiments. Equal amounts of RNA (2 μg) were hybridized overnight at 45°C with a molar excess of ^{32}P-labeled mouse PRL-R and H4 histone riboprobes. Samples were treated with RNAse A (40 μg/ml) and T1 (2 μg/ml), incubated with proteinase K (150 μg/ml), extracted with phenol-chloroform, and precipitated with ethanol. Protected fragments were run on a 6% polyacrylamide/8 M urea gel. Autoradiograms were analyzed with a Biorad GS-700 Imaging Densitometer. H4 histone was used as an internal standard for RNA quantitation.

In Situ Hybridization

Pituitary and brain cryostat sections (10 μm) were prepared from WT and $D_2R^{-/-}$ mice. Ten adult (4- to 14-month-old) females of both genotypes were used for the analysis of hormone expression. TRH and VIP expression in the hypothalamus was analyzed in three WT and three $D_2R^{-/-}$ adult females. In situ hybridization was performed as previously described (Baik et al., 1995). The specificity of the in situ hybridization results was confirmed by the use of sense strand riboprobes, which showed no detectable signals. The number of GH and β-TSH positive cells was estimated by counting clusters of grains, on toluidine-blue counterstained nuclei, five times higher than background. For GH cells, numbers (± SD) are given as profile densities of the number of cells/unit, each unit corresponding to 0.1 mm^2. Two hundred different fields were counted from six serial sections from four WT and four $D_2R^{-/-}$ pituitaries. For β-TSH the number (± SD) of positive cells was evaluated on entire sections, due to the low density of this cell type; 24 sections from four different pituitaries of each genotype were used. Numbers are estimates of profile densities and, as such, have the caveat of being offset by changes in the volume of the pituitaries. Data were analyzed by the Student's *t*-test.

Hormone Analysis

Blood samples were centrifuged at 4000 rpm for 15 min. Sera were removed and stored at −20°C. PRL was measured by a mPRL RIA kit, obtained from Dr. A. F. Parlow (Pituitary Hormones and Antisera Center, UCLA Medical Center, CA). GH and β-TSH RIA kits were from Amersham (UK) and the estrogen RIA kit was from Biomerieux (France).

Immunoblotting and Immunoprecipitation Analysis

PRL-R content in pituitaries from six animals of both sexes and genotypes was analyzed in three independent experiments. Pituitaries were rapidly dissected, frozen, and homogenized in 100 μl of lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM sodium orthovanadate, and 50 mM NaF). Lysates were centrifuged (13,000 rpm for 30 min at 4°C), and protein concentration was estimated by Bradford. PRL-R immunoprecipitations were performed using JAK2 antibodies. One milligram of total proteins in 1 ml of lysis buffer was precleared with preimmune rabbit serum and then incubated for 2 hr at 4°C with 2 μl of anti-mouse JAK2 polyclonal antibody (UBI, NY). Immunoprecipitates were collected with protein A-sepharose (Pharmacia) and resolved on SDS-PAGE. After blotting, filters were probed with U5 anti-rat PRL-R antibody (Interchim, France) or PT-66 anti-phosphotyrosine monoclonal antibody (Sigma). Signals were revealed by enhanced chemiluminescence (ECL; Amersham, UK) and quantified with a Biorad GS-700 Imaging Densitometer.

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