INTRODUCTION

The pituitary gland is an essential regulatory interface integrating signals from the periphery and brain to control the production and secretion of hormones involved in growth, reproduction, behavior and metabolism (Felig et al., 1987; Wilson and Foster, 1992; Gass and Kaplan, 1996; Treier and Rosenfeld, 1996).

The hypothalamus and pituitary gland constitute the main axis of the neuroendocrine system and exhibit a remarkable coordination in temporal and spatial events regulating their development and differentiation (Simmons et al., 1990; Treier and Rosenfeld, 1996).

The pituitary gland originates from Rathke’s pouch, an invagination of the oral ectoderm derived from the most anterior ectoderm of the early embryo (Lancré et al., 1997). The mature pituitary gland consists of five distinct cell types, each defined by the hormone(s) it produces. Thus, the cell types found in the anterior lobe are the thyrotropes, somatotropes, corticotropes, lactotropes and gonadotropes.

SUMMARY

Genetic and molecular approaches have enabled the identification of regulatory genes critically involved in determining cell types in the pituitary gland and/or in the hypothalamus. Here we report that Otx1, a homeobox-containing gene of the Otx gene family, is postnatally transcribed and translated in the pituitary gland. Cell culture experiments indicate that Otx1 may activate transcription of the growth hormone (GH), follicle-stimulating hormone (βFSH), luteinizing hormone (βLH) and α-glycoprotein subunit (αGSU) genes. Analysis of Otx1 null mice indicates that, at the prepubertal stage, they exhibit transient dwarfism and hypogonadism due to low levels of pituitary GH, FSH and LH hormones which, in turn, dramatically affect downstream molecular and organ targets. Nevertheless, Otx1−/− mice gradually recover from most of these abnormalities, showing normal levels of pituitary hormones with restored growth and gonadal function at 4 months of age. Expression patterns of related hypothalamic and pituitary cell type restricted genes, growth hormone releasing hormone (GRH), gonadotropin releasing hormone (GnRH) and their pituitary receptors (GRHR and GnRHR) suggest that, in Otx1−/− mice, hypothalamic and pituitary cells of the somatotropic and gonadotropic lineages appear unaltered and that the ability to synthesize GH, FSH and LH, rather than the number of cells producing these hormones, is affected. Our data indicate that Otx1 is a new pituitary transcription factor involved at the prepubescent stage in the control of GH, FSH and LH hormone levels and suggest that a complex regulatory mechanism might exist to control the physiological need for pituitary hormones at specific postnatal stages.

Key words: Otx1, Cell specificity, Pituitary hormone, Dwarfism, Hypogonadism, Spermiogenesis, Mouse

INTRODUCTION

The pituitary gland is an essential regulatory interface integrating signals from the periphery and brain to control the production and secretion of hormones involved in growth, reproduction, behavior and metabolism (Felig et al., 1987; Wilson and Foster, 1992; Gass and Kaplan, 1996; Treier and Rosenfeld, 1996).
magnocellular neurons are grouped in the paraventricular (PVH) and supraoptic (SO) nuclei, project their axons to the posterior pituitary and release oxytocin (OT) or arginine vasopressin (AVP). The parvocellular neurons are located in the PVH nucleus and release corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH). Additional hypothalamic hormones are growth hormone-releasing hormone (GRH), somatostatin (SS) and gonadotropin-releasing hormone (GnRH). They are synthesized, respectively, in the arcuate (ARH) nucleus, in the anterior periventricular (PVa) nucleus and in scattered neurons at the level of the organum vasculosum of the lamina terminalis (OVLT region) (Mason et al., 1986; Swanson, 1987).

Genetic and molecular approaches have contributed remarkably towards identifying the genes functionally involved in development of the neuroendocrine axis. A large number of transcription factors are required to establish and/or maintain specific cell types in the pituitary gland and the neuroendocrine hypothalamus (Simmons et al., 1990; Treier and Rosenfeld, 1996). Most of them are homeobox-containing genes belonging to different gene families. A large family of POU domain factors has been cloned and classified (He et al., 1989). Members of this family, such as Pit1 and Brn2, have been extensively studied to clarify their role in the coordinate development of the hypothalamic-pituitary axis (Bodner et al., 1988; Ingraham et al., 1988; Li et al., 1990; Nakai et al., 1995; Schonemann et al., 1995).

Other transcription factors belonging to the LIM and Ptx homeodomain families have recently been shown to be required for control of transcription of hormone-coding genes, often exhibiting cooperation between them and/or with specific cofactors (Bach et al., 1995, 1997; Lamonerie et al., 1996; Sheng et al., 1996; Szeto et al., 1996). In vitro and in vivo analyses of these transcription factors support the idea that a complex series of events modulates, in a cell-specific manner, correct expression of terminal targets (Bach, 1997). Failure to have correct activation frequently results in failure in the establishment or survival of specific cell types both in the hypothalamus and pituitary gland. Similarly, during postnatal life, transcription of pituitary hormone genes could be modulated by a similar mechanism involving additional postnatal-specific transcription factors. Therefore, pituitary development might be characterized by an embryonic phase involving the establishment of the pituitary cell types and by a postnatal phase involving the modulation of hormone request in response to growth, differentiation and development of body and organs. In this respect, pituitary postnatal development plays a key role in the early postnatal development of gonadal functions as well as in the maturative events of terminal development of the brain (Wilson and Foster, 1192; Gass and Kaplan, 1996). Furthermore, pituitary postnatal development is likely to be controlled by interactions among regulatory genes, which frequently play a role also during embryonic development.

Here we report that the homeobox-containing gene Otx1 (Simeone et al., 1993), a member of the Otx gene family, which is related to the Ptx family, is expressed in the pituitary gland from birth throughout the adult life and is able to bind the Ptx1 recognition sequence and transactivate the GH, αGSU, βLH, βFSH promoters in cell culture experiments.

In vivo analysis of mice lacking Otx1 reveals transient dwarfism and hypogonadism at the prepubescent stage due to the selective and transient reduction of GH, FSH and LH, which in turn dramatically affects differentiation of downstream organs. In the subsequent 3 months, Otx1−/− mice gradually recover a normal level of pituitary hormones and exhibit catch-up growth as well as restored gonadal function. Our findings provide evidence that Otx1 is a new pituitary transcription factor and suggest that it may play a regulatory function controlling physiological levels of pituitary hormones at a specific postnatal stage.

MATERIALS AND METHODS

Genotypes, growth curves and body and organ weights

Genotypes of Otx1+/+, Otx1+/− and Otx1−/− were determined as previously described (Acampora et al., 1996).

To determine growth curves, the weights versus postnatal age of ten litters (on C57BL6/DBA2 F1 background) were plotted. Fourteen adult stage surviving Otx1−/− (7 males and 7 females), 18 Otx1+/− (9 males and 9 females) and 41 (18 males and 23 females) Otx1+/+ mice were followed essentially as described (Baker et al., 1993). Weight was determined every day in the first postnatal month, every 5 days until the 4th month and every 15 days in the following months. Mean values were determined and selected postnatal stage-points were shown in Fig. 3B. No relevant differences were observed comparing Otx1+/− (not shown) and Otx1+/+ mice. Organ weight was determined by dissecting and weighing fresh organs (brain, testis, kidney and ovary) from mice asphyxiated by CO2. For each sex, a variable number (n=5-7) of Otx1−/− and Otx1+/− were killed at the same postnatal stages indicated in the graph reporting the growth curve.

Radioimmunoassays

Eight male and eight female Otx1−/− and Otx1+/+ mice were killed at each time point indicated in the graphs. Freshly collected pituitaries and testes were weighed and homogenized in 1 ml of the following buffer (10 mM Tris HCl at pH 7.4, 50 mM NaCl, 1% Aprotinin, 1% PMSF and 5 mM EDTA). Four organs were analyzed as single samples and four were pooled. The homogenate was then centrifuged and the supernatant (cytosol) was stored at −80°C until assay. The proteins were measured by the BioRad protein assay reagent. Blood samples were collected in microtubes without anticoagulant. After coat formation the samples were centrifuged and the serum was aspirated and stored at −20°C until assay. Pituitary hormones, IGF1, testosterone and total T4 were determined from the cytosol of organs (pituitary hormones, testosterone and total T4) and serum samples (IGF1), and their levels were normalized for milligram of total proteins. The percentages reported in the graphs were obtained by comparing mean values deduced for Otx1+/+ and individual values for Otx1−/− samples at each time reported. Mean values of Otx1+/− were considered as 100%. GH, FSH, LH, PRL and TSH were measured by specific radioimmunoassay kits (rat GH, rat FSH, rat LH, rat PRL and rat TSH kits, respectively) from Amersham International, UK. IGF1 was measured by a radioimmunoassay kit from Nichols Institute Diagnostics, San Juan Capistrano, CA, USA. Total testosterone was measured with a radioimmunoassay kit from Nichols Institute Diagnostics, San Juan Capistrano, CA, USA. Total T4 was measured with a radioimmunoassay kit from Nichols Institute Diagnostics, San Juan Capistrano, CA, USA. Total T4 was measured with a radioimmunoassay kit from Nichols Institute Diagnostics, San Juan Capistrano, CA, USA. Total T4 was measured with a radioimmunoassay kit from Nichols Institute Diagnostics, San Juan Capistrano, CA, USA.

Immunohistological detection of pituitary hormones

Eight Otx1−/− pituitary glands (four for each sex) at p25 and at 4 months of age were analyzed for hormone content and compared to Otx1+/+ glands at the same stages. Antisera specific for the pituitary hormones were kindly provided by the National Hormone and
Pituitary Program at the NIH (Bethesda, MD): mouse GH antiserum raised in monkey; rat TSH and PRL antiserum raised in rabbit; rat FSH and LH antiserum raised in guinea pig. The rat ACTH antiserum was a rabbit polyclonal Ab purchased from Peninsula Laboratory. The secondary antibodies were purchased from Sigma and Jackson Immunoresearch and used according to the manufacturers’ instructions.

Anatomical and histological analyses
Testes and ovaries were dissected from mice asphyxiated by CO₂ and photographed. For histology, pituitaries, testes and ovaries were fixed in 4% paraformaldehyde/phosphate buffered saline, paraffin embedded, sectioned and stained with haematoxylin-eosin.

RNase protection and RT-PCR assays
For RNase protection experiments, total RNA was extracted from carefully dissected pituitary glands. RNase protection assays were performed using the procedure and the Otx1 probe as previously described (Simeone et al., 1993). The Otx1 RNase protected fragment was 395 bp long. For RT-PCR experiments, total RNA was purified from three dissected pituitary glands and from three brains for each genotype shown, then DNase treated and converted to single-stranded cDNA. cDNA samples were used as templates to amplify GRH (Frohman et al., 1989) and GnRH (Mason et al., 1986) in a standard semiquantitative 20-cycle PCR reaction. Denaturation, annealing and elongation were at 95°C, 60°C and 72°C, respectively. The GRH and GnRH oligo primers are listed below.

RNA probes, in situ hybridization and detection of cell death
In situ hybridization probes for GRH (Frohman et al., 1989), GnRH (Mason et al., 1986), GRHR (Lin et al., 1992), and GnRHR (Tsutsumi et al., 1993) correspond to RT-PCR products of 354 bp, 261 bp, 426 bp and 591 bp, respectively. The primers used in the PCR reactions were the following:

- GRH1: 5'-TCAGTGGGACCTGAGCAGAAC-3';
- GRH2: 5'-ATCCCTGCAAGATGCTCTCCA-3';
- GnRH1: 5'-ATCTCTCAAACTGATGGGCCG-3';
- GnRH2: 5'-TTCTTCTGGCTGCTTCTCTC-3';
- GRH-R1: 5'-GAATTCTTCTCCTACGCGC-3';
- GRH-R2: 5'-CCACACAGCAGCTGAAGTGG-3';
- GnRH-R1: 5'-TCCTTCTGTTGAAGCAGCG-3';
- GnRH-R2: 5'-ATTCAGCTGTAGTTGGCTGG-3'.

Embryos, pituitaries and testes were fixed in 4% paraformaldehyde/phosphate-buffered saline, paraffin embedded and sectioned (Hogan et al., 1994). In situ hybridization was performed as previously described (Hogan et al., 1994). To detect apoptotic cells, the sections were processed following the TUNEL method as described (Gavrieli et al., 1992).

Gel retardation assays
Nuclear microextracts were prepared as described (Therrien and Drouin, 1993) from 300,000 L cells transfected with 20 μg of either control vector or a CMV-Otx1 expression vector (Simeone et al., 1993). Binding reactions, oligonucleotides probes (CE3 element of the rPOMC promoter) and unlabelled competitors have been described previously (Lamonerie et al., 1996). HeLa cells were transfected as previously described (Simeone et al., 1993).

Cell culture and transfection assays
African green monkey kidney fibroblast-like CV-1 cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and transfected as previously described (Lamonerie et al., 1996). Transcriptional activating properties of Otx1 were tested on ~320 bp rat GH, ~1.7 kb mouse tGSU, ~800 bp bovine βLH, ~2.4 kb bovine βFSH, ~480 bp rat POMC and ~3 kb rat PRL pituitary hormone promoters.

Western blot analysis
Crude extracts of pituitaries and 10 days post coitum (d.p.c.) embryos were obtained by lysis in 8 M urea in the presence of 5 mM Tris pH 8 and 0.5% β-mercaptoethanol. 50 μg of pituitary extracts, 10 μg of embryo extracts, 2 μg of nuclear extract from HeLa cells transfected with 20 μg of a CMV-Otx1 expression vector (Simeone et al., 1993) and 10 μg of untransfected HeLa cell extract were electrophoresed and transferred to nitrocellulose in a standard western blot assay and hybridized to a 1:250 diluted anti-OTX1 antibody.

RESULTS

Otx1 is expressed postnatally in the pituitary gland
Most of the transcription factors controlling development and activation of pituitary hormone genes are homeobox-containing factors belonging to different gene families. Among these, members of the POU, LIM and Ptx1/Otx1 classes play a relevant role (He et al., 1989; Bach et al., 1995; Lamonerie et al., 1996; Treier and Rosenfeld, 1996). To gain insight into a possible role of Otx1 in pituitary development, we wondered whether it was expressed in the pituitary during embryonic development and postnatal life. By in situ hybridization, Otx1 expression was undetectable above the background level during embryonic and fetal pituitary development (Fig. 1A-D and data not shown), while it was detected in a uniformly widespread pattern in the postnatal pituitary until the adult stage (4 months after birth) (Fig. 1E-G). No signal was detected with the Otx1 sense strand (Fig. 1H). The Otx1 expression was then confirmed by RNase protection experiments on RNA extracted from purified pituitary glands at different postnatal ages (Fig. 1I). Moreover, by using an anti-OTX1 antibody, it was shown that Otx1 mRNA was also translated both at the weaning and at the adult stages (Fig. 1J). However, it is noteworthy that the amount of the pituitary OTX1 protein appeared to be ~30-fold less than that detected in head extracts from 10.5 d.p.c. wild-type embryos (Fig. 1J and see also Materials and Methods).

Otx1 binds to the promoter and transactivates specific pituitary hormone genes
To support the possibility that Otx1 might be involved as a transcription factor in pituitary physiology, we tested its ability to bind pituitary hormone promoter sequences and/or transactivate their transcription. It had been reported previously that the Ptx1 gene product was able to bind a target sequence originally identified as the CE3 element at ~302 bp of the rat POMC promoter (Lamonerie et al., 1996). Related sequences are also present in one or more copies in the promoter region of other pituitary hormone genes (Tremblay et al., 1998) at the following positions: ~70 bp and ~220 bp of the mouse tGSU promoter; ~92 bp of the bovine βLH promoter; ~52 bp, ~710 bp, ~1209 bp, ~1409 bp, ~1426 bp of the bovine βFSH promoter; ~124 bp and ~217 bp of the rat GH promoter, and ~27 bp of the rat PRL promoter. Since the Ptx1 and Otx1 homeodomains are related to each other and both have bicoid-type DNA recognition (Driever and Nüsslein-Volhard, 1989; Simeone et al., 1993; Lamonerie et al., 1996), we tested the ability of Otx1 gene product to bind to this sequence. Among the putative homologous target sequences listed above, we used for binding experiments an oligonucleotide including the CE3...
element of the rat POMC promoter (see also Materials and Methods) (Tremblay et al., 1998). L cells were transfected with a CMV-\textit{Otx1} expression vector and nuclear extracts were used for the binding reaction. \textit{Otx1} showed strong binding to this target and it was competed specifically by a 200-fold molar excess of the cold wild-type probe, but not by a mutant oligonucleotide that destroys the \textit{Ptx1} consensus binding site (Fig. 2A).

We then investigated the possibility that besides being able to bind, \textit{Otx1} was also able to drive the transcription of pituitary hormone genes in CV-1 cells. Therefore, CMV-\textit{Otx1} was cotransfected with luciferase reporters for the GH, \textit{βFSH}, \textit{βLH}, POMC, \textit{αGSU} and PRL promoters (see also Materials and Methods) and the luciferase activity monitored, revealing that the \textit{Otx1} gene product was able to promote the transcription of \textit{βLH}, \textit{βFSH} and \textit{αGSU} glycoprotein by 3- to 5-fold. It did not activate POMC and PRL promoters, and enhanced GH transcription at a low level (Fig. 2B).

Emerging data suggest that cooperative interactions among...
different transcription factors result in synergistic effects on the promoter of target genes (Lamonerie et al., 1996; Treier and Rosenfeld, 1996; Bach et al., 1997; Poulin et al., 1997; Tremblay et al., 1998). For this reason, we tested whether the GH promoter might be activated synergistically by Otx1 and Pit1. Interestingly, Otx1 and Pit1 showed a cumulative effect on transcription of the GH promoter (Fig. 2B).

These data indicate that Otx1 can bind to a specific target sequence in the promoter region of pituitary hormone genes, and is able to transactivate βFSH, βLH and αGSU transcription. Moreover, the finding that Otx1 and Pit1 may cooperate in modulating GH transcription reinforces the idea that specific combinations of different transcription factors may define the correct transcriptional rate of their pituitary target hormone genes.

Mice lacking Otx1 show pituitary impairment

Otx1 null mice were previously generated to study the role of Otx1 in brain development (Acampora et al., 1996). We showed that Otx1−/− mice suffer from epilepsy and impairment of proper brain and sense organ functions. However, Otx1−/− mice are particularly relevant to determine whether the Otx1 gene product also plays a role in pituitary development and/or physiology, thus supporting the findings obtained by in situ hybridization and transfection experiments. Otx1−/− mice were generated and analyzed in the hybrid C57BL6/DBA2 (B6/D2) and pure 129/Sv genetic backgrounds. However, since the percentage of death increased up to 80% when the homozygous mutation was analyzed in the 129/Sv pure background while death was ~25% in the B6/D2 hybrid background, we decided to perform our analysis in the B6/D2 background. Our first observation was that, although body weight and size of newborn Otx1−/− mice were unaltered as compared to those of Otx1+/+ (Fig. 3A) and Otx1+/− (data not shown), around postnatal day 7 (p7) Otx1−/− mice exhibited an increasing dwarfism, with peak reduction in both size and body weight at around p30 (Fig. 3A). During the following 3 months, Otx1−/− mice gradually increased in size, weight and rate of growth, becoming indistinguishable from Otx1+/+ (Fig. 3A). The recovery was maintained during the following months. However, it is worth noting that even in 129/Sv pure background the Otx1−/− surviving mice showed the same phenotypic behaviour. This observation was further supported by measuring postnatal growth and comparing body and organ weights of Otx1−/− to those of Otx1+/+ and Otx1+/− mice from birth until 1 year of age. Ten litters containing in total 14 surviving Otx1−/−, 18 Otx1+/+, and 41 Otx1+/− mice were followed to define the growth pattern; in addition, a variable number of mice for each genotype (n=4-7) and for each stage analyzed were killed to define body and organ weights. A comparison of the postnatal body weight curves (Laird et al., 1965; Baker et al., 1993) of Otx1−/− and Otx1+/+ showed that the rate of growth decreased progressively in the first month of age (Fig. 3B), but increased in the following months to become indistinguishable from wild type by about the fourth month (Fig. 3B). Similarly, weight of body and tested organs (kidney, ovary, testis), with the exception of brain, decreased both in size and weight, recovering the normal phenotype later, while the brain showed 20-25% reduced weight from birth with no subsequent recovery (Acampora et al., 1996) (data not shown and see Materials and Methods). Data from Otx1+/+ matched perfectly those of wild type (data not shown).

To rule out the possibility that competition during milk sucking was responsible for growth retardation, 10 Otx1−/− mice housed alone with their mothers were followed. An identical transient reduction of growth rate as well as of body and organ weight was detected (data not shown). Also, the presence of expected amounts of milk intake was verified by the analysis of stomach content in Otx1−/− compared to Otx1+/+ mice (data not shown).

To test for pituitary involvement in generating this phenotype, we performed radioimmunoassays and immunohistological studies of pituitary hormones. Radioimmunoassays were performed separately on male and female Otx1−/− and Otx1+/− mice to detect pituitary levels of GH, FSH, LH, TSH and PRL either in single pituitary glands (n=4/stage) or in pituitary pools (4 pituitaries/pool/stage).
Between p20 and p30, while TSH and PRL revealed only a small transient reduction, FSH and LH levels appeared transiently reduced with a peak at p20, and then recovered to a normal level (Fig. 7A,B). At 4 months of age, they had recovered to a normal level (Fig. 7A).

In Fig. 4, only the percentages obtained by comparing mean Otx1+/+ and Otx1−/− values deduced from single pituitaries have been reported; however, data from the pools were found to be very similar to the mean values (data not shown). Between p20 and p30, a remarkable reduction of 70-80% was found in Otx1−/− mice of both sexes for GH, FSH and LH, but not for TSH and PRL (Fig. 4A,B) which were only slightly reduced (10-15%). As observed for body weight, pituitary levels of GH, FSH and LH also gradually increased and, at 4 months of age, they achieved their normal levels in Otx1−/− mice of both sexes (Fig. 4A,B). However, it should be noted that the GH level already appeared remarkably reduced at p10 and then recovered to a normal level at the end of the first postnatal week.

Immunohistological studies of pituitary GH, FSH, LH, TSH, PRL and ACTH were performed at p25 and 4 months of age to support radioimmunoassays data. Analyses at p25 and 4 months of age showed changes that parallel the results of the radioimmunoassays. At p25, only GH, FSH and LH appeared to be strongly reduced (Fig. 5A-C) while, at 4 months of age, they were comparable in Otx1+/+ and Otx1−/− pituitaries (Fig. 5G-I). In sharp contrast, immunohistological detection of Otx1+/+ and Otx1−/− glands (Fig. 5D-F).

An important question is whether the reduction in GH, FSH and LH is due to a recoverable loss of somatotropic and gonadotrophic cells or to a transient reduction in hormone synthesis without loss of cells. We investigated this in two ways: first, by performing pituitary histological analysis using in situ hybridization with the cell-type-restricted receptors for hypothalamic somatotropic (GRH) (Lin et al., 1992) and gonadotrophic (GnRH) factors (Tsutsumi et al., 1992), and, second, by analysing pituitary cell apoptosis (Drewett et al., 1993). Histology of Otx1−/− pituitary glands revealed no abnormalities as compared to those of wild type both at p20 and p40 days (Fig. 6A-D and data not shown). Similarly, receptors for GnRH (GnRHR) (Fig. 6E-H) and GRH (GRHR) (Fig. 6I-L) were transcribed in the mutant pituitary glands with an expression pattern indistinguishable from that of wild type. Finally, we studied the level of cell death at postnatal days 10 and 20, and after 4 months of age in Otx1−/− and Otx1+/+ mice. As previously reported (Drewett et al., 1993), no or very rare apoptotic cells were detected with the TUNEL method in Otx1+/+ pituitaries at p20 and p40. Otx1−/− pituitaries at p20 and p40 also did not reveal any significant increase in apoptotic cell number either (data not shown). Altogether these findings suggest that it is the ability to synthesize hormones rather than the total cell number that is altered in the pituitaries of Otx1−/− mice.

**Pituitary deficit of GH, FSH and LH transiently affects downstream molecular and organ targets**

Perturbation in pituitary GH, FSH and LH levels was expected to affect body growth, differentiation and size of testis and ovary (Kumar et al., 1997) as well as the synthesis of their molecular mediators (Felig et al., 1987; Wilson and Foster, 1992; Gass and Kaplan, 1996).

We already showed that body weight was affected transiently as a possible consequence of GH deficiency. Since insulin-like growth factor 1 (IGF1) mediates many of the effects of GH postnatally (Baker et al., 1993), we determined the serum levels of IGF1 by radioimmunoassay. IGF1 was transiently reduced both in males and females with a minimum around p30, and then recovered to a normal level (Fig. 7A,B). Similarly, we examined development of the gonads (ovary and testis) and, as well as the levels of tissue testosterone (testis) since these parameters would be expected to be affected by the severe reduction in FSH and LH (Wilson and Foster, 1992; Gass and Kaplan, 1996). In Otx1−/− mice, testosterone levels were markedly reduced around p30 but, by 4 months of age, they had recovered to a normal level (Fig. 7A).

Both the ovary and testis were strongly impaired in size and differentiation, paralleling the reduction in LH and FSH (Parvinen, 1982; Kumar et al., 1997). The size reduction of testis was hardly detectable at p10 and reached its maximal decrease at p30 (Fig. 8A-C); subsequently, normal size was recovered (Fig. 8D,E). At p1, p5, and p10, testicular histology appeared very similar to that of the wild type (data not shown); at p20, the seminiferous tubules of mutant mice did not show an open lumen (Fig. 8F,F′) and at p30 they were strongly or completely depleted of presumptive secondary spermatocytes (Fig. 8G,G′,J,J′), suggesting a selective loss of differentiating sperm cells but not of spermatogonial precursors. This was also supported by the subsequent reappearance of differentiating...
spermatocyte cells which become mature sperm at 4 months of age (Fig. 8I,K,K'). At this stage, all the $Otx1^{-/-}$ males were fertile despite a reduced frequency of mating. Thus, the transient reduction in gonadotropic hormones appeared to block the committed spermatogenesis but preserved spermatogonial precursor cells, which restored a normal spermatogenesis paralleling the recovering of hormonal levels.

To gain insight into the possibility that apoptotic cell death could contribute to the specific loss of differentiating spermatocytes, the TUNEL procedure (Gavrieli et al., 1992) was performed on p10, p20 and p30 $Otx1^{+/+}$ and $Otx1^{-/-}$ testes. $Otx1^{+/+}$ testes revealed normal apoptotic pattern that is higher at p10 (Fig. 8L) as compared to p20 (Fig. 8M) and p30 (Fig. 8N) where only a few apoptotic cells are normally identified.
Hypothalamic GRH and GnRH expression are unaltered in Otx1\(^{-/-}\) mice

The release of pituitary hormones is under the control of hypothalamic hormones (Swanson 1986, 1987). In Otx1\(^{-/-}\) mice, impairment of GRH and GnRH expression might contribute to the phenotype by affecting the release of pituitary GH, LH and FSH.

We therefore performed in situ hybridization with GRH (Frohman et al., 1989) and GnRH (Mason et al., 1986) probes to test their correct expression. No obvious alteration appeared by comparing Otx1\(^{+/+}\) (Fig. 9A,B) and Otx1\(^{-/-}\) (Fig. 9C,D) expression patterns at p30, or at earlier and later stages (data not shown).

The expected numbers of neurons producing GRH and GnRH were identified in the arcuate nucleus (Fig. 9A,C) and at the level of the organum vasculosum of the lamina terminalis (Fig. 9B,D), respectively. Moreover, semiquantitative RT-PCR assays confirmed no quantitative differences in their levels between the mutant and wild-type brains at p30 (Fig. 9E,F). Therefore, we conclude that, in Otx1\(^{-/-}\) mice, the survival, differentiation and position of GnRH- and GRH-expressing cells involved in the formation of the hypothalamo-pituitary axis were apparently unaffected. Taken together with the normal expression of the pituitary receptors for these hypothalamic hormones, our results suggest that signaling between hypothalamus and pituitary is intact in Otx1\(^{-/-}\) mice. Pituitary responsiveness to these signals might be altered by the absence of pituitary Otx1.

**DISCUSSION**

Development of the anterior pituitary gland results in the specification of distinct cell types characterized by the ability to synthesize and release trophic hormones. Somatotrophic, gonadotropic, thyrotropic, lactotropic and corticotropic cell types secrete GH, FSH, LH, TSH, PRL and ACTH, respectively. To be active, βFSH, βLH and βTSH need to heterodimerize with the αGSU subunit (Kendall et al., 1995). Similarly, hypothalamic development leads to the specification of distinct cell types with neuroendocrine functions (Swanson, 1986, 1987). Recent data defining the genes involved in differentiation and survival of these cellular components, have provided insight into understanding the molecular mechanism underlying the coordinate establishment of the hypothalamo-pituitary axis. These genes encode transcription factors frequently characterized by the presence of a homeodomain and belonging to different gene families including the LIM, POU and PTX/OTX classes (He et al., 1989; Bach et al., 1995; Sheng et al., 1996). In vitro and in vivo analyses indicate that a complex mechanism of transcriptional interactions, spatially and temporally regulated, defines the coordinate development of the hypothalamus and pituitary gland. Failure in these interactions results in marked impairment of pituitary and hypothalamic functions (reviewed in Treier and Rosenfeld, 1996; Sharp and Morgan, 1996).

In this study, we present in vitro and in vivo evidence demonstrating the involvement of one member of the Otx gene family – namely Otx1 (Simeone et al., 1993) – in the establishment of proper pituitary function. Our findings support a regulatory role for Otx1 in modulation of specific pituitary hormone (GH, FSH and LH) expression at a specific postnatal stage. This unprecedented finding suggests the existence of a mechanism modulating synthesis of specific hormones at specific postnatal stages.

This mechanism may involve a direct transcriptional action of Otx1 on the promoters of the affected hormones (GH, αGSU, βLH, βFSH), but it may also involve other targets in the gonadotroph and somatotroph cells of the pituitary. Since differentiation of those lineages does not appear to be affected and since a basal level of hormones is produced in the Otx1\(^{-/-}\)
mice, a possible role for Otx1 might be to mediate signals that regulate the level of hormone-coding mRNAs and the synthesis of the encoded proteins. These signals may be elicited by GRH or GnRH or by other growth-related elements. An intriguing aspect of our observation is the fact that transcription factors of the Ptx and Otx subfamilies recognize similar DNA target sequences (Simeone et al., 1993; Lamonerie et al., 1996; Szeto et al., 1996; Tremblay et al., 1998), and that Ptx1 and Ptx2 are expressed in most pituitary lineages, in particular in somatotroph and gonadotroph cells (Tremblay et al., 1998). Ptx1 is the most highly expressed of these genes followed by Ptx2 and then Otx1 (Tremblay et al., 1998, and data not shown). Yet, the Otx1 knock-out has a dramatic effect during the prepubertal period. The unique activity of Otx1 during this period might reflect a specific interaction of Otx1, but not of the related Ptx factor(s) with a coregulator of transcription in the somatotroph and gonadotroph cells. Alredy, it appears that Ptx1 interacts specifically with Pit1 in the somatolactotroph lineage to synergistically activate the PRL promoter (Szeto et al., 1996; Tremblay et al., 1998), that it synergistically interacts...
Indeed, our findings strongly suggest that \( \text{Otx}1 \) is involved in normal pituitary physiology. \( \text{Otx}1^{+/−} \) mice showed unprecedented reductions in GH, FSH and LH synthesis as well as in the level and differentiation of their molecular and organ targets. In particular, impairment in gonadal development such as the reduction of testis size as well as the block in folliculogenesis are expected from decreased FSH and are consistent with the phenotype of FSH null mice (Kumar et al., 1997). An important point of this in vivo study was to assess whether \( \text{Otx}1 \) affects pituitary cell survival or the level of hormone transcription or both. Our data provide evidence favouring the second possibility. This conclusion is supported primarily by the absence of obvious differences between \( \text{Otx}1^{−/−} \) and \( \text{Otx}1^{+/+} \) in histology of the pituitary gland, the expression patterns of the receptors for hypothalamic somatotropic and gonadotropic releasing factors, and the failure to detect an increased level of apoptosis in mutant pituitary glands. The downstream molecular and organ targets for GH, FSH and LH, but not for TSH, were also impaired, thus confirming a specific effect of \( \text{Otx}1 \) on somatotropic and gonadotropic functions. Moreover, we showed that both GRH and GnRH hypothalamic neurons were apparently unaffected. Thus, although the \( \text{Otx}1^{−/−} \) brain was impaired in different areas, including the cortex, mesencephalon and cerebellum, a possible involvement of \( \text{Otx}1 \) in hypothalamic control of somatotropic and gonadotropic pituitary hormones was not evident.

An unprecedented feature of our in vivo analysis is the fact that most of the impaired functions described here had recovered by the adult stage (4 months), when \( \text{Otx}1 \) is normally still transcribed and translated and, therefore, likely to have a regulatory role. Nevertheless, after the prepubescent stage, \( \text{Otx}1^{−/−} \) mice began to gradually recover from their abnormalities showing at 4 months of age normal levels of GH, FSH and LH, which paralleled the restored body weight, differentiation and size of both testis and ovary, as confirmed also by their sexual fertility, and normal levels of downstream molecular targets such as testosterone and IGF1. Although we are unable to explain the mechanism underlying this recovery, we report this observation as a possible example of temporal-restricted competence in hormonal regulation of specific cell lineages by the \( \text{Otx}1 \) transcription factor. This recovery appears similar to the ‘catch-up growth’ (Boersma and Wit, 1997) described in children with delayed growth and puberty, also called constitutional delay in growth and adolescence, CDGA (Horner et al., 1978). In conclusion, our observations have revealed a complex mechanism of gene regulation for hormone synthesis defined by different and temporal-restricted combinations of specific transcriptional factors and cofactors. This combinatorial code could underlie different temporal windows of competence, each defining physiological levels of pituitary hormones.

We are deeply indebted to L. Lania and P. Sassone-Corsi for advice and criticism, and to C. Goodyer and H. Gudy for suggestions on the manuscript. We thank R. Di Lauro for helpful discussions and A. Secondulfo for manuscript preparation. R. Maurer, D. Gordon, J. Nilson, M. Karin and G. Rosenfeld are thanked for kindly providing the \( \beta \)-FSH, \( \alpha \)-GSU, \( \beta \)-LH, GH and PRL promoter constructs, respectively. This work was supported by grants from the Italian Telethon Program to A. S. and the Italian Association for Cancer Research (AIRC) to A. S. and G. C., the Centre National de la
REFERENCES


