

Progressive impairment of developing neuroendocrine cell lineages in the hypothalamus of mice lacking the *Orthopedia* gene

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Development of the neuroendocrine hypothalamus is characterized by a precise series of morphogenetic milestones culminating in terminal differentiation of neurosecretory cell lineages. The homeobox-containing gene *Orthopedia* (*Otp*) is expressed in neurons giving rise to the paraventricular (PVN), supraoptic (SON), anterior periventricular (aPV), and arcuate (ARN) nuclei throughout their development. Homozygous *Otp*^{-/-} mice die soon after birth and display progressive impairment of crucial neuroendocrine developmental events such as reduced cell proliferation, abnormal cell migration, and failure in terminal differentiation of the parvocellular and magnocellular neurons of the aPV, PVN, SON, and ARN. Moreover, our data provide evidence that *Otp* and *Sim1*, a bHLH-PAS transcription factor that directs terminal differentiation of the PVN, SON, and aPV, act in parallel and are both required to maintain *Brn2* expression which, in turn, is required for neuronal cell lineages secreting oxytocin (OT), arginine vasopressin (AVP), and corticotropin-releasing hormone (CRH).

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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). By integrating signals from the periphery and brain, this neuroendocrine system controls the synthesis and secretion of the hormones required for body growth, behavior, reproduction, and metabolism (Felig et al. 1987; Wilson and Foster 1992; Gass and Kaplan 1996; Treier and Rosenfeld 1996). 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 that produce the thyroid-stimulating hormone (TSH), growth hormone (GH), adrenocorticotropic hormone (ACTH), prolactin (PRL), follicle-stimulating hormone (FSH), and luteinizing hormone (LH), respectively (Swanson 1986, 1987; Felig et al. 1987; Wilson and Foster 1992; Gass and Kaplan

1996). The neuroendocrine hypothalamus consists of two distinct neuronal populations; the magnocellular and parvocellular neurons (Swanson 1986, 1987; Sharp and Morgan 1996). The magnocellular neurons are grouped in the paraventricular (PVN) and supraoptic (SON) nuclei, project their axons to the posterior pituitary, and release oxytocin (OT) and arginine vasopressin (AVP). The parvocellular neurons project to the median eminence (ME), in which they release hypophysiotrophic hormones that are conveyed to the anterior pituitary by the hypophyseal-portal vascular system. The parvocellular neurons located in the PVN nucleus release corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH). Centered in ventrally contiguous cell groups in the anterior periventricular (aPV) or the arcuate nuclei (ARN) of the hypothalamus are hypophysiotrophic neurons synthesizing somatostatin (SS) or growth-hormone-releasing hormone (GHRH), which impart the principal inhibitory and stimulatory regulation of GH, respectively.

Parvocellular and magnocellular precursor neurons of the PVN and SON are generated in the mouse embryos between embryonic day 10 (E10) and E12 in the supraoptic/paraventricular (spv) area (Karim and Shopper 1980). A fraction of these neurons remains in a medial position

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to give rise to the PVN, whereas the residual portion migrates laterally to where they reach the final destination between E13.5 and E14.5 and form the SON (Karim and Sloper 1980). Between E13.5 and E14.5, neuroendocrine neurons start to synthesize hormones and this event defines the terminal differentiation of the neuroendocrine hypothalamus.

Genetic and molecular approaches have contributed remarkably toward identifying a number of genes functionally involved in the development of the neuroendocrine axis and most of them are homeobox-containing genes belonging to different gene families (Treier and Rosenfeld 1996). Members of the *LIM*, *PTX*, and *OTX* classes are required for proper transcriptional control of pituitary hormones and often exhibit 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; Acampora et al. 1998).

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 studied extensively 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; Simmons et al. 1990; Nakai et al. 1995; Schonemann et al. 1995).

Recently, the bHLH-PAS transcription factor *Sim1* has been shown to play a crucial role in the development of parvocellular and magnocellular neurons of the PVN, SON, and aPV as well as in the maintenance of *Brn2* expression (Michaud et al. 1998). Consequently, neuroendocrine impairments detected in *Brn2*^{-/-} mice (Nakai et al. 1995; Schonemann et al. 1995) are present also in mice lacking the *Sim1* gene (Michaud et al. 1998). Finally, mice lacking the homeodomain factor *Gsh-1* do not synthesize GHRH in the ARN (Li et al. 1996) indicating that *Gsh-1* is required for proper development of a specific neuroendocrine cell lineage different from those requiring *Sim1* and *Brn2* (Li et al. 1996; Treier and Rosenfeld 1996).

Orthopedia (*Otp*) is a highly conserved homeodomain-containing factor that is transcribed during murine embryonic development in a segment-like expression pattern including the anterior hypothalamus (AH), spv, retrochiasmatic, and ventral tuberal areas (Simeone et al. 1994; Avantaggiato et al. 1995) that give rise to the aPV, PVN, SON, and ARN (Puelles and Rubenstein 1993; Rubenstein et al. 1994; Alvarez-Bolado et al. 1995).

Here, we report that in mice lacking *Otp*, aPV, PVN, and SON were absent, whereas ARN was impaired but present. *Otp*^{-/-} mice failed to express CRH, TRH, AVP, OT, and SS, whereas they retained a normal expression of GHRH in the ARN. Furthermore, we provide evidence that *Otp* is required for multiple stage-specific cellular functions leading to the establishment of the neuroendocrine hypothalamus.

Results

Generation of *Otp* null mice

To decipher the *Otp* role in the development of the neu-

roendocrine hypothalamus, *Otp* null mice were generated (Fig. 1A; see Materials and Methods). Homologous recombination events result in the deletion of part of the first exon and the entire second exon including the methionine and most of the homeodomain, and in the fusion of the *lacZ* coding sequence to the *Otp* 5'-untranslated region. HM-1 homologous recombinant ES cell clones were identified by PCR and confirmed by Southern blot analysis (Fig. 1B; Materials and Methods). *Otp*^{+/-} mice genotyped by allele-specific PCR reactions (Fig. 1C) were healthy and fertile, and their intercross yielded offspring in the expected mendelian ratio. *Otp*^{-/-} pups died either at birth, or within the first two postnatal days (P1–P2) without apparent malformations. To assess the complete inactivation of the gene, the absence of the OTP protein was confirmed by a Western blot assay on E13.5 *Otp*^{-/-} embryonic extracts with an anti-OTP polyclonal antibody (Fig. 1D).

To verify whether the *lacZ* expression was correctly driven by *Otp*-transcription control elements, transcripts from the normal (probe b in Fig. 1A) and mutated (probe c in Fig. 1A) alleles were compared with each other. In particular, in heterozygotes at E13.5 (Fig. 1G,H), E15.5 (Fig. 1I,J), and P1 (Fig. 1K,L), the expression patterns of the normal and mutated alleles were totally overlapping, thus indicating that *lacZ* was a reliable marker to follow *Otp*-expressing cells in *Otp*^{+/-} and *Otp*^{-/-} mutants. Similarly, at E10 and E12.5, the β-galactosidase (β-gal) staining (Fig. 1E,F) was very similar or identical to the *Otp* RNA distribution revealed by whole-mount in situ hybridization experiments (data not shown). On this basis, although all the analyzed *Otp*^{+/-} brains were hybridized with both the allele-specific probes, we have chosen here to show only *lacZ* expression.

Anatomo-histological and molecular abnormalities in *Otp*^{-/-} mice

Anatomo-histological analysis of *Otp*^{-/-} brains at postnatal day (P1) showed evident abnormalities in the hypothalamus. Nissl staining of histological sections revealed that as compared with *Otp*^{+/-} (Fig. 2A,C,E,G), in *Otp*^{-/-} brains, the presumptive PVN and SON showed hypocellularity and absence of proper morphological features (arrows in Fig. 2A',C'), whereas ME (arrow in Fig. 2E') and the posterior lobe of the pituitary gland (Fig. 2G') were extremely hypoplastic. Surrounding structures such as the suprachiasmatic nucleus (SCN) and the zona incerta (ZI) appeared morphologically normal (Fig. 2A'; data not shown). To reinforce these observations, in situ detection of calbindin D28K (calb) by a polyclonal antibody (α-calb) was determined. α-calb stained restricted neuronal populations including neurons of the SON, PVN (Fig. 2B,D), as well as projections from the PVN and aPV to the ME (Fig. 2F; Enderlin et al. 1987; Nakai et al. 1995). According to the morphological analysis, calb immunoreactivity resulted essentially abolished in the presumptive SON, PVN, and ME of *Otp*^{-/-} brains (Fig. 2B',D',F'). Noteworthy, in *Otp*^{-/-} brains, the proliferat-

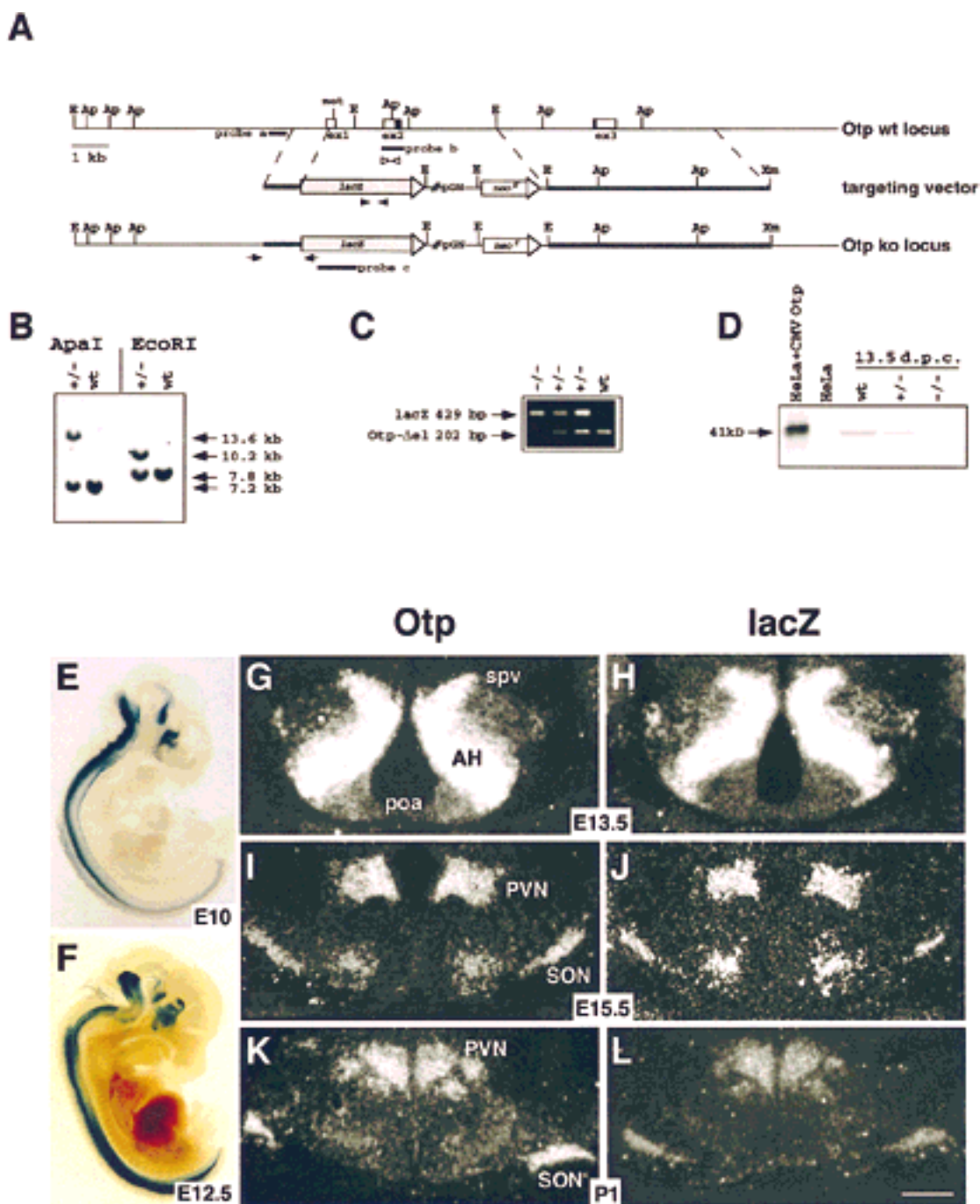


Figure 1. Targeted replacement of *Otp* with the *lacZ* gene. (A) Schematic representation of the wild-type *Otp* allele, targeting vector, and recombined allele. E, Ap and Xm stand for *EcoRI*, *ApaI*, and *XmnI*. The locations of probes a, b, and c used for Southern blot analyses (probes a and c) and in situ hybridizations (probes b and c) are indicated. The arrows in the third line indicate the position of the primers used to identify homologous recombinant clones. (B) Southern blot analysis of one targeted ES cell line and wild-type HM-1 ES cells showing expected hybridization pattern of *ApaI* and *EcoRI*-digested genomic DNA samples with probe a (A). Only the 13.6-kb band with *ApaI* and the 10.2-kb band with *EcoRI* were detected with the *lacZ* internal probe c (data not shown). (C) Genotyping of littermates from the intercross of heterozygotes by a PCR reaction amplifying fragments specific for the deleted region of *Otp* (*Otp* Δ el) (202 bp) and *lacZ* (429 bp), with the primers indicated as open and filled arrowheads, respectively. (D) Western blot analysis of the OTP protein in transfected HeLa cells and embryonic extracts. (E–L) β -gal staining of *Otp*^{+/-} embryos at E10 (E) and E12.5 (F) and in situ hybridization on adjacent sections of *Otp*^{+/-} brains at E13.5 (G,H), E15.5 (I,J), and P1 (K,L) with allele-specific probes (probes b and c in A) revealing no difference between the wild-type and the targeted locus. (spv) Supraoptic/paraventricular area; (AH) anterior hypothalamus; (PVN) paraventricular nucleus; (poa) anterior preoptic area; (SON) supraoptic nucleus. For sections in G–L, scale bar corresponds to 50 μ m (L).

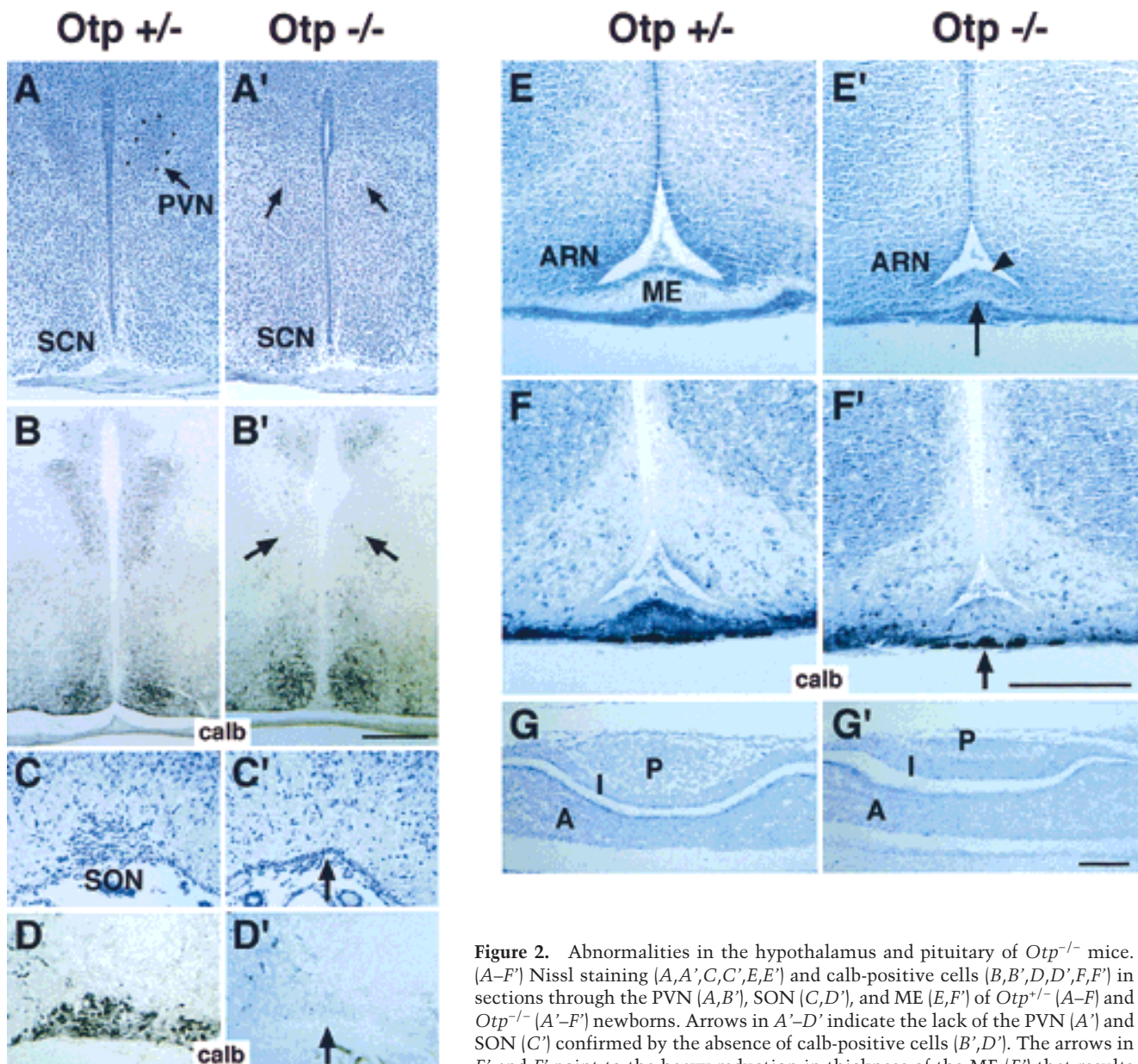


Figure 2. Abnormalities in the hypothalamus and pituitary of *Otp*^{-/-} mice. (A–F′) Nissl staining (A,A′,C,C′,E,E′) and calb-positive cells (B,B′,D,D′,F,F′) in sections through the PVN (A,B′), SON (C,D′), and ME (E,F′) of *Otp*^{+/-} (A–F) and *Otp*^{-/-} (A′–F′) newborns. Arrows in A′–D′ indicate the lack of the PVN (A′) and SON (C′) confirmed by the absence of calb-positive cells (B′,D′). The arrows in E′ and F′ point to the heavy reduction in thickness of the ME (E′) that results poorly stained with α-calb antibody (F′). Note that the neuroepithelium adjacent to the presumptive ME results in increased thickness (arrowhead in E′).

(G,G′) As compared with *Otp*^{+/-} (G), the posterior lobe of the pituitary gland is strongly hypoplastic in *Otp*^{-/-} mice (G′). Abbreviations as in the previous figure; (ME) median eminence; (SCN) supra-chiasmatic nucleus; (ARN) arcuate nucleus; (A) I, P anterior, intermediate, and posterior lobes of the pituitary. Scale bars, 50 μm. Scale bar in B′ refers to A and B′; scale bar in F′ to C–F′, and scale bar in G′ to G–G′.

ing neuroepithelium adjacent to the presumptive ME was found to be increased in thickness (arrowhead in Fig. 2E′).

To further characterize the defects in the neuroendocrine hypothalamus, we wondered whether *lacZ* expression was affected. In *Otp*^{+/-} brains, *lacZ* is expressed all along the PVN (Fig. 3A–C), in the aPV (Fig. 3A), the anterior hypothalamic area (AH) (Fig. 3A), the SON (Fig. 3B), the ARN (Fig. 3D), a narrow domain of cells adjacent to the ARN (arrowheads in Fig. 3D), and the premam-

millary area (pMA) (Fig. 3D). In *Otp*^{-/-} brains, an evident reduction in the number and position of cells expressing *lacZ* was observed (Fig. 3A′–D′). *lacZ*-positive cells were detected in a territory that normally did not express *Otp* (Fig. 3A′–C′), and no transcripts were found in the presumptive area corresponding to the aPV, PVN, and SON (Fig. 3A′–C′). In contrast, *lacZ* expression in the ARN was retained, even though in an apparently reduced number of cells (Fig. 3D′). These data, therefore, suggest that differentiation, proliferation, and migration of

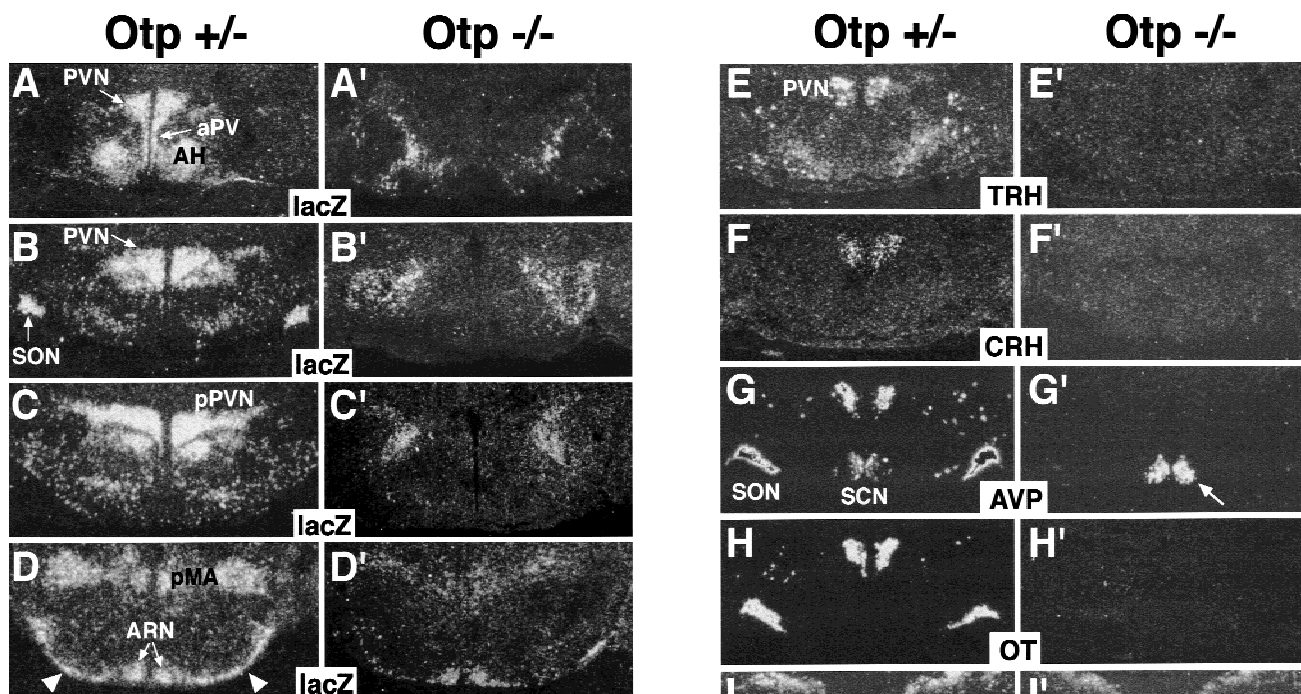


Figure 3. Abnormal *lacZ* expression and lack of neuroendocrine hormones in *Otp*^{-/-} brain. (A–C′) Coronal sections of *Otp*^{+/-} (A–C) and *Otp*^{-/-} (A′–C′) brains at P1 showing that *lacZ* is expressed in *Otp*^{+/-} brain all along the PVN (A–C), aPV (A), AH (A), and SON (B), whereas in *Otp*^{-/-} brain, *lacZ* transcripts disappear from the presumptive areas corresponding to the PVN (A′–C′), aPV (A′), AH (A′), and SON (B′) and are detected in a misplaced domain that normally never expresses *Otp* (A′–C′). (D, D′) In sections through the ARN of *Otp*^{+/-} (D) and *Otp*^{-/-} (D′) brains, *lacZ* is expressed in the ARN, in a narrow domain adjacent to the ARN (arrowheads) and in a region possibly including the premammillary nucleus. Note the remarkable reduction in *lacZ* expression in *Otp*^{-/-} brains. (E–K′) In *Otp*^{+/-} brain (E–K), TRH (E) is detected in the PVN and in scattered neurons, CRH (F) in the PVN, AVP (G) in the PVN, SON, and SCN, OT (H) in the PVN and SON, SS (I, J) in numerous areas and among them in the aPV (I) and ARN (J) and GHRH in the ARN (K), whereas in *Otp*^{-/-} brain (E′–K′), TRH (E′), CRH (F′), AVP (G′), and OT (H′) are undetectable in the PVN and SON, SS is not transcribed in the aPV (I′) and ARN (J′), whereas GHRH (K′) appears unaffected. Also note that in *Otp*^{-/-} mice, TRH expression totally disappears in the hypothalamus; AVP expression is retained in the SCN (G′), and SS-positive neurons are not identified in the area surrounding the ARN (J′). Abbreviations as in the previous figures; (aPV) anterior periventricular nucleus; (pMA) premammillary nucleus. Scale bar, 50 μm.

Otp^{-/-} expressing cells might be heavily perturbed. In this context, we wondered whether the ability to transcribe OT, AVP, TRH, CRH, GHRH, and SS was still retained. As compared with *Otp*^{+/-} (Fig. 3E–K), in *Otp*^{-/-} (Fig. 3E′–K′) brains, TRH, CRH, OT, and AVP were not expressed in the PVN (TRH and CRH) (Fig. 3E′, F′) or in the PVN and SON (AVP and OT) (Fig. 3G′, H′); SS was absent in the aPV (Fig. 3I′) and in an extended area including the ARN and surrounding territory (Fig. 3J′), whereas GHRH was correctly transcribed (Fig. 3K′). Moreover, it is noteworthy that TRH-positive neurons totally disappeared in *Otp*^{-/-} brains (Fig. 3E′; see below), and AVP transcription was not affected in the SCN, in which *Otp* was not expressed (Fig. 3G′). Noteworthy, in some (two of seven) of the *Otp*^{-/-} brains analyzed, the AVP expression in the SCN appeared increased.

Therefore, these findings prove that *Otp* codes for a critical function required for the correct development of the neurosecretory system and indicate that *Otp*-expressing cells that are normally committed to giving rise to neuroendocrine nuclei appear reduced in number and placed in an abnormal position.

Failure in terminal differentiation of the neuroendocrine hypothalamus in *Otp*^{-/-} embryos

Terminal differentiation of major neuroendocrine nuclei was marked by the expression of hormones between E13.5 and E14.5. Therefore, we studied whether the onset of hormone expression was correctly established at E13.5 (data not shown) and E14.5 (Fig. 4). As compared with *Otp*^{+/-} embryos (Fig. 4A–M), the expression of TRH

(Fig. 4B',G'), AVP (Fig. 4C',H'), OT (Fig. 4D',I'), and CRH (Fig. 4E') was abolished in *Otp*^{-/-} mutants; SS (Fig. 4K') transcripts were not detected in the ARN or in the adjacent area, whereas the GHRH was expressed normally (Fig. 4L'). The same result was obtained at E13.5 for the hormones already expressed at this stage (data not shown). Thus, in *Otp*^{-/-} embryos, neuropeptide expression failed to be established in the PVN and SON, whereas it resulted partially impaired in the ARN.

To correlate lack of hormones to the *Otp* expression domain, the distribution of *lacZ* transcripts was investigated. In *Otp*^{+/-} embryos, *lacZ* was coexpressed with the hormones (Fig. 4A,F,I) and interestingly, at the level of the posterior PVN (pPVN), *lacZ* (Fig. 4F) was detected in two streams of cells deriving from it and likely migrating in a latero-ventral direction to form the posterior SON and expressing AVP and OT (Fig. 4H,I), or in a more medio-ventral direction and expressing TRH (Fig. 4G). In *Otp*^{-/-}, at the level of the preoptic area (poa) *lacZ* transcripts were detected in the AH, but not in the presumptive PVN (arrow in Fig. 4A'), whereas they were found at the level of the presumptive pPVN (Fig. 4F'). Nevertheless, in this area, OT/AVP and TRH-positive neurons were not identified and an abnormal *lacZ* expression was detected (Fig. 4F'). At the level of ARN and adjacent re-

gions, a moderate reduction of both *lacZ* expression and calb-positive cells was observed (Fig. 4J',M').

Early development of hypothalamic neuroendocrine precursor cells and *Otp* expression in *Sim1* mutants

To study how and when the abnormal phenotype was generated, a detailed analysis was performed from E10.5 to E14.5. As mentioned previously, *Brn2* and *Sim1* genes are expressed and required for the correct development of aPV, PVN, and SON (Nakai et al. 1995; Schonemann et al. 1995; Michaud et al. 1998). In addition, calb is an early marker of most of the neuronal cells committed to form PVN and SON, even before the onset of the hormone transcription (Nakai et al. 1995). For these reasons, their expression patterns were analyzed and compared with that of *lacZ* in adjacent sections. The first abnormality was detected at E10.3, when a smaller domain of β -gal staining was observed in *Otp*^{-/-} embryos (data not shown). At E11.5, in *Otp*^{-/-} embryos, the *lacZ* and *Sim1* domains were narrowed in both the spv and AH primordia and *Brn2* expression was slightly toned down in the postmitotic areas in which it was coexpressed with *lacZ* (data not shown).

At E 12.5 in *Otp*^{-/-} embryos, the hypothalamic im-

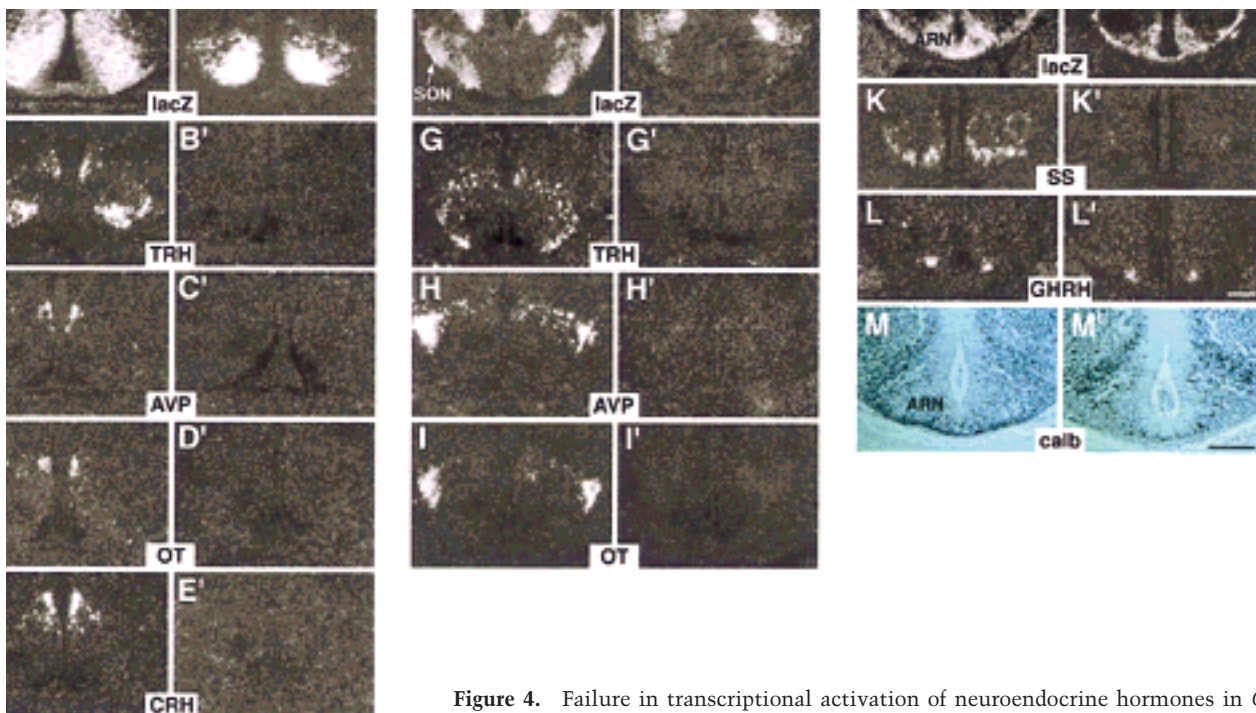


Figure 4. Failure in transcriptional activation of neuroendocrine hormones in *Otp*^{-/-} embryos. (A–L') Frontal sections through the hypothalamus of E14.5 *Otp*^{+/-} (A–L) and *Otp*^{-/-} (A'–L') embryos showing that at the level of the poa area (A–E') *lacZ* transcripts disappear from the presumptive PVN primordium (arrow in A') and TRH (B'), AVP (C'), OT (D'), and CRH (E') hormone transcripts are not detected; at the level of the pPVN (F–I') *lacZ* is abnormally transcribed (F') and no expression appears for the TRH (G'), AVP (H'), and OT (I'); and at the level of the ARN (J–L') no macroscopic abnormalities are detected in the distribution of *lacZ* (J') and GHRH (L') transcripts, whereas SS-positive neurons disappear (K'). (M, M') As compared with *Otp*^{+/-} (M), a considerable reduction in the number of calb-positive cells is observed in the ARN and surrounding areas of *Otp*^{-/-} embryos (M'). Abbreviations as the previous figures. Scale bars, 50 μ m. Scale bar in L' refers to dark fields, and in M' to M, and M'.

(F–I') *lacZ* is abnormally transcribed (F') and no expression appears for the TRH (G'), AVP (H'), and OT (I'); and at the level of the ARN (J–L') no macroscopic abnormalities are detected in the distribution of *lacZ* (J') and GHRH (L') transcripts, whereas SS-positive neurons disappear (K'). (M, M') As compared with *Otp*^{+/-} (M), a considerable reduction in the number of calb-positive cells is observed in the ARN and surrounding areas of *Otp*^{-/-} embryos (M'). Abbreviations as the previous figures. Scale bars, 50 μ m. Scale bar in L' refers to dark fields, and in M' to M, and M'.

pairments began to be evident. In *Otp*^{+/-} embryos, *Brn2* expression (arrow in Fig. 5B,E) overlapped with that of *lacZ* in the spv primordium at the level of both the poa and posterior preoptic (pop) areas (arrow in Fig. 5A,D), and numerous migrating calb-positive cells were identified within the *lacZ* domain (Fig. 5C,F). Interestingly, in *Otp*^{-/-} embryos, *Brn2* was not transcribed in the spv (arrow in Fig. 5B',E'), and the number of calb-positive cells resulted markedly reduced (Fig. 5C',F'). This latter observation is in agreement with the lack of *Brn2* expression, because a very similar reduction in the number of calb-positive cells has been already reported in mice lacking *Brn2* (Nakai et al. 1995).

In comparison with *Otp*^{+/-} at E13.5 (Fig. 6A-H), in *Otp*^{-/-} embryos at the level of the poa region, *lacZ* and *Sim1* transcripts were not uniformly distributed in the spv and AH (Fig. 6A',C') and in sections crossing the pop area were abolished in the early presumptive SON area (arrow in Fig. 6E',G') and heavily reduced in the spv (arrowheads in Fig. 6E',G'), whereas they were apparently unaffected in the AH (Fig. 6E',G'). *Brn2* continued to be expressed only in the periventricular neuroepithelium (Fig. 6B',F') and very few and disorganized calb-positive cells were still detected in restricted regions coexpressing *lacZ* and *Sim1* (Fig. 6D', and circled area in H'). No calb-positive cells were identified in the presumptive SON region (arrow in Fig. 6H'). Finally, at E14.5, the major abnormalities described at P1 were evident. As compared with *Otp*^{+/-} (Fig. 6I-P), in *Otp*^{-/-} embryos at the level of the poa region, the expression of *lacZ* and *Sim1* disappeared from the presumptive PVN region (arrow in Fig. 6I',K'). *Brn2* expression and calb-positive cells were undetectable in the PVN (Fig. 6J and arrow in L').

At the level of the presumptive pPVN, *lacZ* and *Sim1* were cotranscribed in an abnormal domain (Fig. 6M',O'); *Brn2* was not expressed (Fig. 6N'), and a few calb-positive cells (Fig. 6P') were misplaced in a small area in which *lacZ* and *Sim1* were transcribed (circled area in Fig. 6P' and arrowhead in M',O'). To investigate whether *Otp*

might be downstream of *Sim1*, *Otp* expression was studied in *Sim1*^{-/-} embryos. At E12.5, as compared with *Sim1*^{+/-} (Fig. 7A,A'), in *Sim1*^{-/-} embryos, *Otp* (Fig. 7B') was stably transcribed in the territory expressing the *Sim1* null alleles (Fig. 7B), thus indicating that the *Otp* expression does not require the SIM1 gene product to be maintained.

In summary, these data suggest that (1) between E10 and E12, reduced proliferating activity and/or increased cell death might be responsible for the reduction in the extent of *lacZ* and *Sim1* domains; (2) subsequently, between E13 and E15, at the level of the poa region, the PVN cells became disorganized, and then at E14.5, probably died as revealed by the disappearance of four different markers (*lacZ*, *Brn2*, *Sim1*, and calb); (3) at the level of the pop area, at least a fraction of *lacZ*-positive cells destined to form the pPVN and two distinct cell populations normally secreting AVP/OT or TRH (Fig. 4F-I) still survived, but failed to both properly differentiate (Fig. 4F'-I') and migrate; (4) *Otp* was required for maintenance of *Brn2* expression in the spv, whereas it was not required to control *Sim1* transcription; (5) *Sim1* is not required for *Otp* expression in *Sim1*^{-/-} mutants.

Apoptosis and cell proliferation in *Otp*^{-/-} embryos

On the basis of previous considerations, apoptosis and cell proliferation were studied in *Otp* mutants and *Otp* expression was analyzed in *Sim1*^{-/-} embryos. Apoptosis was investigated at E10.5, E11.5, E12.5, E13.5, E14.5, E15.5, and P1. Three mutants for each stage were analyzed. In situ detection of apoptotic cells by the TUNEL method (Gavrieli et al. 1992) revealed no significant difference between *Otp*^{+/-} and *Otp*^{-/-} embryos at all the stages analyzed in both the areas expressing *lacZ* or not (data not shown), thus indicating that even though *Otp*^{-/-} embryos were affected by increased cell death, this was not due to apoptosis. The gradual reduction of the *lacZ* expression domain observed from E13 onward

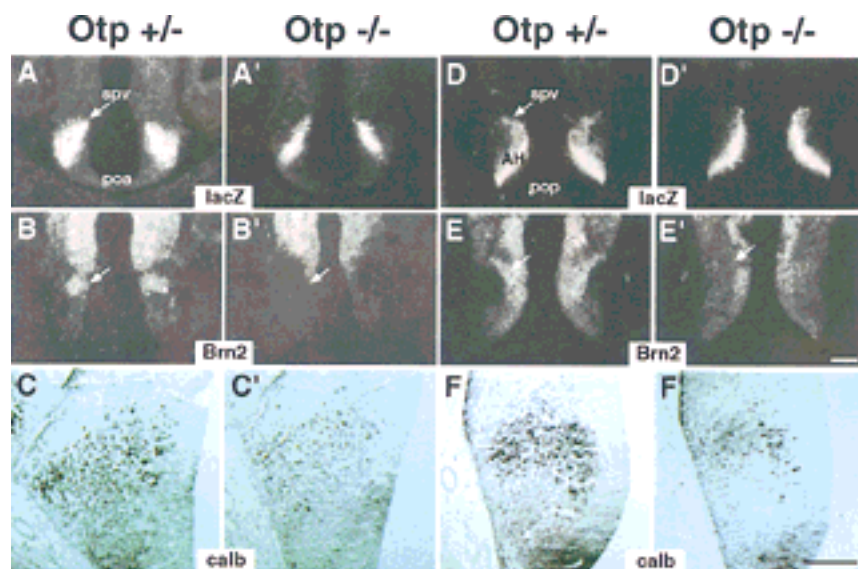
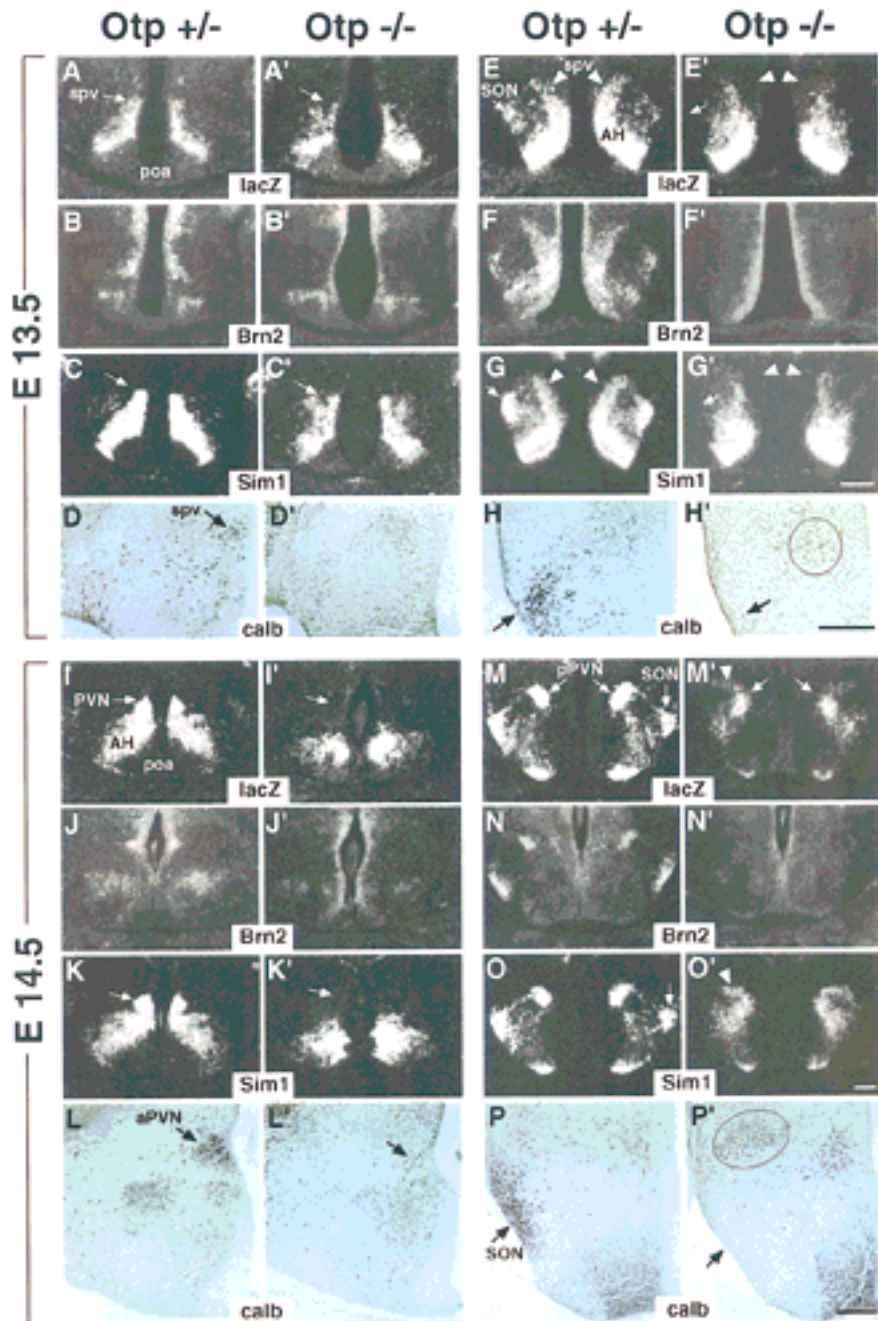


Figure 5. Abnormalities in *Otp*^{-/-} embryos at E12.5. (A-F') *Otp*^{+/-} (A-F) and *Otp*^{-/-} (A'-F') embryos hybridized with the indicated *lacZ* (A,A',D,D') and *Brn2* (B,B',E,E') probes or processed for detecting calb-positive cells (C,C',F,F') in sections adjacent to those hybridized with radioactive probes and photographed at a higher magnification. Note that *Otp*^{-/-} embryos show narrowed expression domains of *lacZ* (A',D'); lack of *Brn2* transcripts in the spv (arrow in B',E'); and, finally, a remarkable reduction in the number of calb-positive cells (C',F'). Abbreviations as in the previous figures; in C,C',F,F' the third ventricle is at right. Scale bars, 50 μ m. Scale bar in E' refers to dark fields and in F' to bright fields.

Figure 6. Abnormalities in *Otp*^{-/-} embryos at E13.5 and E14.5. (A–P') Adjacent frontal sections of *Otp*^{+/-} (A–P) and *Otp*^{-/-} (A'–P') embryos at E13.5 (A–H') and E14.5 (I–P') are hybridized with the indicated *lacZ*, *Brn2*, and *Sim1* or stained for detecting calb-positive cells. At E13.5 in sections through the poa area of *Otp*^{-/-} embryos, *lacZ* (A') and *Sim1* (C') transcripts are disorganized and poorly condensed in the spv and in sections through the pop area; the *lacZ* and *Sim1* expression disappears from the presumptive SON (cf. arrows in E,G with arrows in E',G') and is heavily affected in the spv region (cf. arrowheads in E,G with arrowheads in E',G'). At E14.5 in sections through the poa area, *lacZ* and *Sim1* expression is not detected in the PVN primordium (arrow in I',K') and at the level of pPVN, a fraction of migrating *lacZ*- (M') and *Sim1*- (O') positive cells is still identified. In E13.5 and E14.5 *Otp*^{-/-} embryos, only sporadic calb-positive cells are identified in the spv/PVN primordium (cf. D with D' and arrow in L with arrow in L') and in the presumptive SON (cf. arrow in H,P with arrow in H',P', respectively). Note that the circled group of calb-positive cells (P') that is coincident with the *lacZ* and *Sim1* expression (arrowhead in M',O') may, possibly, correspond to residual cells that would be committed to migrate and form the SON. Finally, *Brn2* expression in the spv, PVN, SON, and pPVN of *Otp*^{+/-} embryos (B,F,I,N) is undetectable in *Otp*^{-/-} mutants (B',F',I',N'). Note that calb-staining sections (D,D', H,H',L,L',P,P') are adjacent to those hybridized with radioactive probes, but are photographed at a higher magnification. Abbreviations as in the previous figures and in L,L',P,P', the third ventricle is at right. Scale bars, 50 μm. Scale bar in G' refers to A–C' and E–G'; scale bar in O' to I–K' and M–O'; scale bar in H' to D,D',H,H'; scale bar in P' to L,L',P,P'.



led us to strongly suspect that survival of a conspicuous number of *lacZ*-positive cells was compromised during mid-late gestation.

Cell proliferation was investigated by both a short pulse or a longer exposure to bromodeoxyuridine (BrdU) and subsequent comparison between BrdU- and *lacZ*-positive cells in *Otp*^{+/-} and *Otp*^{-/-} embryos. Because a thinner *lacZ* domain was already evident in E10.5 *Otp*^{-/-} embryos (Fig. 7F; data not shown) and neuronal precursors of SON and PVN were generated massively between E10 and E12 (Karim and Sloper 1980), pregnant mice at E10.3, E11.3, and E12.5 were injected with BrdU and sacrificed after 1 hr. Within the proliferating neuroepithelium, *lacZ* was transcribed only in those neurons

that were close to the mantle zone (Fig. 7C–H) and BrdU-positive cells were detected approximately in the same number in *Otp*^{+/-} and *Otp*^{-/-} embryos (Fig. 7, cf. C',D',E' with F',G',H'), thus indicating that abnormality in proliferative activity measured in 1 hr of exposure to BrdU was not evident in *Otp*^{-/-} embryos. Nevertheless, to investigate the fate and the number of proliferating neuroblasts detected over a longer temporal window, embryos were injected with BrdU at E11.3 and sacrificed at E12.5. The number of BrdU-positive cells amassed within the *lacZ* domain at E12.5 was remarkably decreased in *Otp*^{-/-} embryos and their position was abnormal (Fig. 7, cf. I,I' with K,K'). Moreover, when BrdU was injected at E10.3 and the embryos analyzed at E12.5, a decrease in

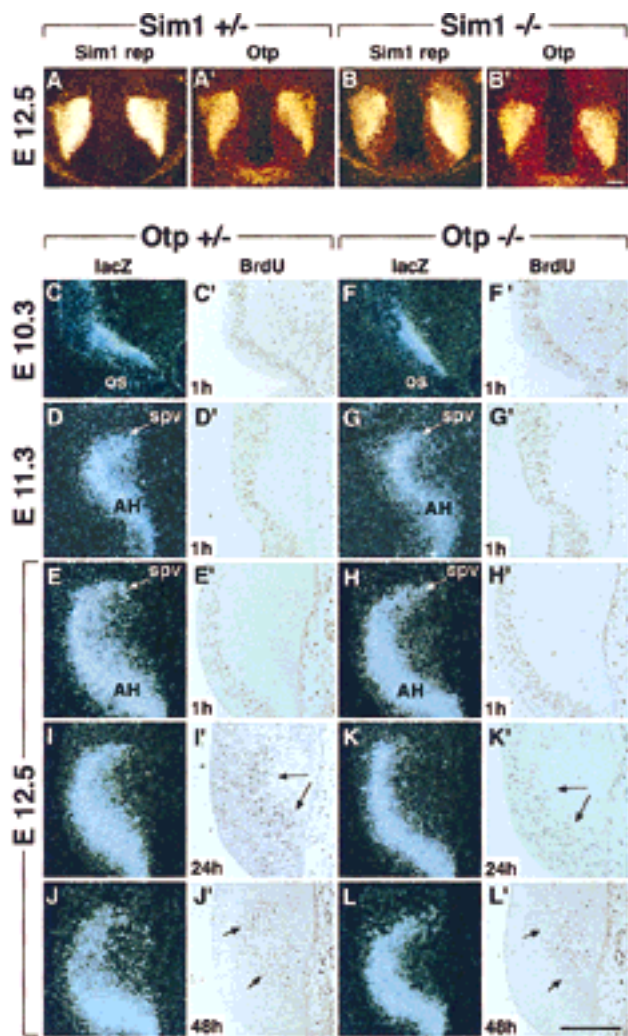


Figure 7. *Otp* expression in *Sim1*^{-/-} mutants and cell proliferation in *Otp*^{-/-} embryos. (A–B') *Otp* expression (A',B') was compared with that of the *Sim1* reporter (*Sim1* rep) (A,B) in E12.5 *Sim1*^{+/-} (A,A') and *Sim1*^{-/-} (B,B') embryos, showing no obvious difference between the two genotypes, thus indicating that *Sim1* was not required for *Otp* expression. (C–H') *lacZ* expression (C–H) and BrdU-positive cells (C'–H') labeled 1 hr after the injection were compared in *Otp*^{+/-} and *Otp*^{-/-} embryos at E10.3 (C,C',F,F'), E11.3 (D,D',G,G'), and E12.5 (E,E',H,H') showing no obvious difference between the two genotypes. Note that *lacZ* was expressed in the proliferating neuroepithelium only in the portion close to the mantle zone. (I–L') At E12.5, comparison between *lacZ* expression (I–L) and BrdU-positive cells (I'–L') detected 24 (I',K') and 48 hr (J',L') after a single injection of BrdU reveals that, as compared with *Otp*^{+/-} (I,I',J,J'), in *Otp*^{-/-} (K,K',L,L') embryos, the *lacZ* expression domain was thinner (K,L) and the number of BrdU-positive cells (K',L') remarkably reduced. Abbreviations as in the previous figures; (os) optic stalk. Arrows in I', J', K', and L' point to BrdU-positive cells that are diminished in *Otp*^{-/-} embryos. Scale bars, 50 μ m. Scale bar in B' refers to A–B' and in L' to C–L'.

the number of BrdU-labeled cells was observed in *Otp*^{-/-} embryos (Fig. 7, cf. J,J' with L,L'). Therefore, these data indicate that, at least between E10.3 and E12, *Otp* is required for normal proliferating activity and that a de-

creased neuroblast proliferation may contribute to explaining the narrowed domain of *lacZ* expression.

Patterning abnormalities in mice lacking Otp

Comparison between the expression patterns of *Otp* and *Dlx1*, a murine homolog of the *Drosophila Distal-less* (*Dll*) gene (Cohen 1990; Price et al. 1991), has suggested that *Otp* and *Dlx1* might contribute to defining subregionally restricted identities within the hypothalamus (Simeone et al. 1994). In particular, at E12.5, the *Otp* expression in the spv and AH was complementary to that of *Dlx1* in the poa, ventral thalamus (vt), and supra-chiasmatic area (sch) (Bulfone et al. 1993; Simeone et al. 1994; Avantsaggiato et al. 1995). No abnormalities in the *Dlx1* and *lacZ* expression patterns were detected at this stage in *Otp*^{-/-} embryos (data not shown).

Compared with *Otp*^{+/-} at E15.5 and P1, in *Otp*^{-/-} brains, the *lacZ* expression disappeared from the presumptive PVN territory (Fig. 8, cf. the black asterisk in A,C,E,G with the white asterisk in A',C',E',G'), in which it was replaced by that of *Dlx1* (Fig. 8, cf. the white asterisk in B,D,F,H with the black asterisk in B',D',F',H').

Therefore, even though the complementary expression between *lacZ* and *Dlx1* expression domains was maintained, the presumptive PVN area showed expression of *Dlx1*, a gene that normally is never transcribed in the PVN.

Discussion

The neurogenesis of the endocrine hypothalamus is characterized by a sequential series of crucial events including initial commitment to the neuronal fate, neuroblast proliferation, migration of postmitotic neurons to the neuroendocrine nuclei, and terminal differentiation that results in neuropeptide activation and axonal outgrowth. We reported that *Otp* affects all of these events except the initial commitment to the neuronal fate (Fig. 9A,B).

Otp is required for neuroendocrine terminal differentiation

Mice lacking the *Otp* gene die soon after birth and fail in terminal differentiation of neuroendocrine cell types corresponding to parvocellular and magnocellular neurons of the PVN, SON, aPV, and ARN. In particular, TRH, CRH, SS, OT, and AVP neuropeptide transcription and axonal outgrowth fail to be established in *Otp*^{-/-} mutants (Fig. 9A). Failure in terminal differentiation of specific neuroendocrine cell lineages has been reported in *Sim1*^{-/-}, *Brn2*^{-/-}, and *Gsh1*^{-/-} mice (Schonemann et al. 1995; Li et al. 1996; Michaud et al. 1998). In this context, the finding that *Otp* controls not only aPV, PVN, and SON cell types that require *Sim1* or *Sim1* and *Brn2*, but also SS-producing neurons of the ARN, provides further support to the concept that specific combinations of

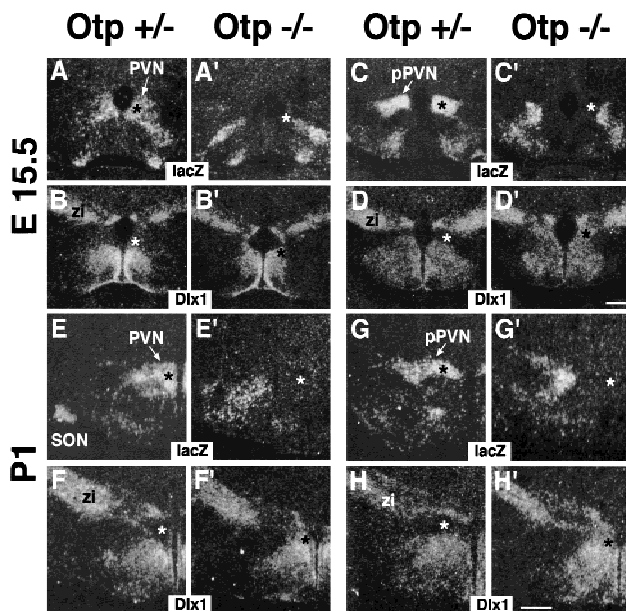


Figure 8. Patterning abnormalities in *Otp*^{-/-} mutants. (A–H') Adjacent frontal sections through the hypothalamus of *Otp*^{+/-} (A–H) and *Otp*^{-/-} (A'–H') at E15.5 (A–D') and P1 (E–H') hybridized with *lacZ* and *Dlx1* showing that in the PVN of *Otp*^{+/-} brain, *lacZ* and *Dlx1* show complementary expression (cf. black asterisk in A,C,E,G with white asterisk in B,D,F,H), where as in the area corresponding to the presumptive PVN of *Otp*^{-/-} mutants, the *lacZ* and *Dlx1* complementary expression was inverted (cf. white asterisk in A',C',E',G' with black asterisk in B',D',F',H'). Abbreviations as in previous figures; (zi) zona incerta. Scale bars, 50 μ m. Scale bar in D' refers to E15.5 and in H' to P1 brains.

regulatory factors are necessary to confer differentiative information that underlie the acquisition of a specific cellular identity (Figdor and Stern 1993; Puelles and Rubenstein 1993; Lamoniere et al. 1996; Sharp and Morgan 1996; Sornson et al. 1996; Treier and Rosenfeld 1996). Moreover, we also reported that the ventricular neuroepithelium adjacent to the ME of *Otp*^{-/-} brain is abnormally increased, suggesting that *Otp* might be required also for processes other than the differentiation of specific neuroendocrine cell lineages.

Cell proliferation, apoptosis and cell migration in *Otp* mutants

The first abnormality detected in *Otp*^{-/-} embryos corresponds to a narrowed *lacZ* expression domain due to a reduced number of cells expressing it. This may be caused by either a decreased cell proliferation or increased cell death. Our findings indicate that a diminished proliferating activity of cells that should transcribe *Otp* is responsible for the lower number of *lacZ*-expressing cells between E10 and E13. Interestingly, between E10 and E13, *lacZ* is not expressed through the entire proliferating neuroepithelium, but is excluded from the ventricular side and maintained in the neuroepithelium

close to the mantle zone. A detailed comparison of *Otp* expression and BrdU-positive cells indicates that the number of BrdU-positive cells labeled at E11.3 and identified at E12.5 within the *lacZ* expression domain results remarkably decreased in *Otp*^{-/-} embryos. Moreover, because the abnormal proliferating activity is not revealed after a BrdU pulse of 1 hr, it can be argued that *Otp* might be required to control the number of cell divisions and/or the length of the cell cycle only in neuroblasts expressing it. However, because a reduction in the number of *lacZ*-expressing cells may also be due to an increased cell death, an exhaustive analysis to detect apoptosis has been performed (see Material and Methods section).

No apoptotic cells are detected at any of the stages analyzed, thus indicating that apoptosis does not contribute to the *Otp*^{-/-} abnormalities. Nevertheless, it cannot be excluded that in *Otp*^{-/-} embryos, cells die for a different reason. In this context, between E13.5 and E14.5, at the level of the *poa*, *lacZ*-positive cells of the *spv* primordium appeared disorganized and then disappeared, leading us to strongly suspect that at least these cells die, even though apoptosis is not revealed. Apoptosis has not been reported in *Sim1*^{-/-} or *Brn2*^{-/-} mutants (Nakai et al. 1995; Schonemann et al. 1995; Michaud et al. 1998), even though it appears not increased in *Sim1*^{-/-} mutants (Michaud J., pers. comm.).

Therefore, although our findings indicate that reduced proliferation is a major abnormality detected in *Otp*^{-/-} embryos, we favor the possibility that a fraction of *lacZ*-expressing cells die from E13 onward. To follow the fate of *Otp*-expressing cells, the *Otp* gene has been replaced with the *lacZ* reporter gene whose expression was correctly driven by the *Otp* promoter elements. In *Otp*^{-/-} brain at P1, the *lacZ* expression domain is heavily affected, as it is abnormal in position and extent. The residual *lacZ*-positive cells, instead of being localized in the presumptive PVN and SON, occupy a ventro-lateral domain in which they are never found in normal embryos. Abnormal migration of *lacZ*-expressing cells is also supported by the abnormal position of both *lacZ* and BrdU-positive cells from E12 onward. Moreover, the finding that *Sim1* and *lacZ* are coexpressed also in *Otp*^{-/-} embryos, provides further evidence that *lacZ*/*Sim1*-positive cells identified in abnormal position correspond to those cells that should be normally fated to form the PVN and SON and that, in the absence of *Otp*, lack this important commitment and fail to properly migrate. This finding suggests that the absence of a single gene function, namely *Otp*, results in the abnormal positioning of cells normally fated to generate hypothalamic neuroendocrine nuclei and suggests that, in these cells, proper migration and differentiation are controlled by the same gene product. In this context, it should be interesting to analyze cell migration in *Sim1* and/or *Brn2* mutant mice. Moreover, understanding the abnormalities of *Otp*^{-/-} mutants might help in the future to define, in more detail, genetic requirement(s) conferring positional value to neuronal precursors of restricted cell lineages (see also below).

In summary, *Otp* plays a role in proliferation and/or

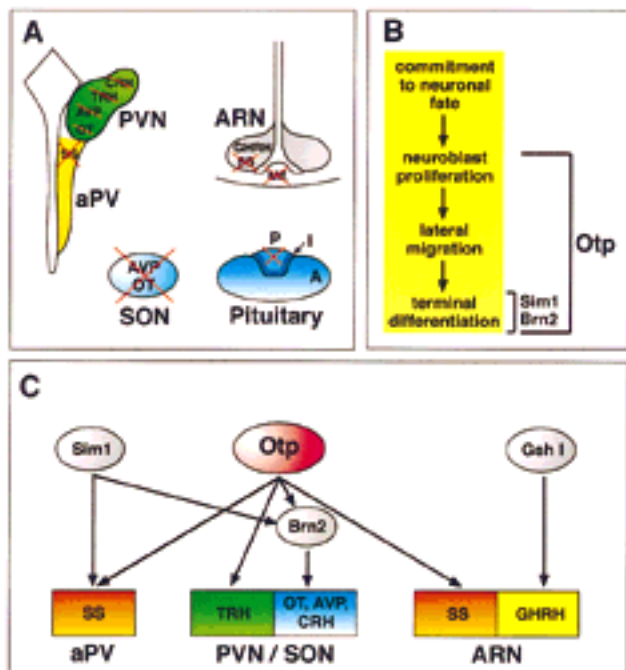


Figure 9. Schematic summary illustrating neuroendocrine impairments, abnormal developmental processes, and genetic interactions deduced from the analysis of *Otp*^{-/-} mice. (A) In wild-type mice magnocellular and parvocellular neurons of the PVN, SON, aPV, and ARN secrete OT, AVP, CRH, TRH, SS, and GHRH neuropeptides. Magnocellular neurons located in the PVN and SON project their axons to the posterior lobe of the pituitary, whereas parvocellular neurons of the PVN, aPV, and ARN project to the ME. In *Otp*^{-/-} mice, failure in terminal differentiation results in morphological disruption of PVN and SON, lack of CRH, TRH, AVP, OT, and SS neuropeptides expression, and impaired axonal outgrowth to the ME and posterior pituitary. (B) Developmental milestones of neuroendocrine hypothalamus are represented by the early commitment to the neuronal fate, neuroblast proliferation, lateral migration of post-mitotic neurons to the area of nuclei formation, and terminal differentiation including neuropeptide expression, axonal outgrowth, and maintenance of cell viability. In *Brn2*^{-/-} and *Sim1*^{-/-} mice, terminal differentiation of cell lineages secreting CRH, AVP, OT (for *Brn2*), and CRH, AVP, OT, TRH, and SS (for *Sim1*) is disrupted. In *Otp*^{-/-} mice, beside the failure in terminal differentiation of CRH, AVP, OT, TRH, and SS cell lineages, there is also an impairment in both neuroblast proliferation, and their subsequent migration, indicating that *Otp* is an important requirement of most of the crucial steps necessary for proper development of the neuroendocrine hypothalamus. (C) *Otp* is required from E12 onward for *Brn2* and neuropeptide expression. Similar findings have been provided in *Sim1*^{-/-} mice. Our data indicate that *Sim1* and *Otp* are largely coexpressed and that *Sim1* and *Otp* expression is unaffected in *Otp*^{-/-} and *Sim1*^{-/-} embryos, respectively, thus providing genetic evidence that they act in parallel and are both required for *Brn2* expression. However, it cannot be argued from our data whether *Sim1* and *Otp* require an additional genetic element to activate SS and TRH expression in the aPV and PVN, respectively. Finally, in the ARN, *Otp* is required only for SS but not for GHRH expression that is controlled by *Gsh1*. These data support the existence of a complex hierarchy of genetic interactions among transcription factors selectively required in specific cell lineages of the developing neuroendocrine hypothalamus.

survival as well as migration and differentiation. Multiple requirements might be the consequence of different cell and stage-specific roles, or, alternatively, all the abnormalities identified might be the consequence of an early and unique role that appears first manifested with abnormal proliferation and reduced cell number and later with impaired migration and failure in terminal differentiation. This aspect is potentially of great interest as it raises the question of whether events underlying neuroendocrine development are hierarchically interdependent or independent.

Further experiments on the basis of cell-restricted and stage-specific inactivation of *Otp* might provide insights into these aspects.

Patterning abnormalities in Otp mutants

Along the hypothalamus, the *Otp*-expression domain is complementary to that of *Dlx1* (Simeone et al. 1994). This observation has suggested that *Otp* and *Dlx1* might contribute to defining specific territorial identities by supplying positional and differentiative information within the secondary prosencephalon or the neuromere D1 (Figdor and Stern 1993; Puelles and Rubenstein 1993). Moreover, it has also been proposed that diencephalic neuromeres may represent discrete units of polyclonal cell population with restricted lineages (Figdor and Stern 1993). Precursor cells of a polyclonal population were generated in the neuroepithelium and migrate to reach their final destination. In this context, cell lineage-restricted expression of regulatory genes might represent a crucial step for cells to both migrate to the final destination and restrict their identity.

In the secondary prosencephalon of *Otp*^{-/-} brain, *lacZ*-positive cells retain *Sim1* expression, but abnormally migrate, disappearing from the presumptive PVN area in which, conversely, *Dlx1*, a gene never expressed in the PVN, is abundantly transcribed, thus suggesting that *Dlx1*-positive cells have replaced PVN-committed cells (positive for *lacZ* and *Sim1* expression) that have abnormally migrated. Finally, the observation that, although in an abnormal position, the *lacZ* and *Dlx1* domains maintain their complementary expression, indicates that even in the absence of *Otp*, *lacZ* and *Dlx1*-expressing cell populations retain a sufficient degree of specific identity and, thereby, do not mix with each other.

Otp acts upstream of Brn2 and in parallel with Sim1

Our data on *Otp* and previous reports on the role of *Brn2*, *Sim1*, and *Gsh1* (Nakai et al. 1995; Schonemann et al. 1995; Li et al. 1996; Michaud et al. 1998) genes support the idea that different combinations of transcription factors act in concert to specify a developmental program leading to the generation of distinct neuroendocrine cell lineages. Analysis of *Brn2* mutant mice revealed that it acts relatively late in neuroendocrine development, being required for terminal differentiation events of CRH, AVP, and OT cell lineages (Fig. 9B,C) (Schonemann et al.

1995; Sharp and Morgan 1996; Treier and Rosenfeld 1996).

Sim1 mutant mice showed a more general effect, as they were impaired in terminal differentiation events leading to the activation of neuropeptides of the PVN and SON as well as of SS in the aPV (Fig. 9B,C; Michaud et al. 1998). Interestingly, from E12.5 onward, *Sim1*^{-/-} mutants gradually lack *Brn2* expression in the dorsal spv primordium indicating that *Sim1* acts upstream of *Brn2* and is required for maintenance of its expression (Fig. 9C; Michaud et al. 1998). Our data reveal a striking similarity with *Sim1* mutant phenotype. Except in the ARN, *Otp* is fully coexpressed in time and space with *Sim1*, and is required for both terminal differentiation of parvocellular and magnocellular neurons of aPV, PVN, and SON and for maintenance of *Brn2* expression. Noteworthy, at E11.5, *Brn2* expression is slightly toned down and, at E12.5, disappears from the entire spv and adjacent territory in which it is coexpressed with *Otp*, thus suggesting that as compared with *Sim1*^{-/-} phenotype, *Otp* may have a more generalized role in controlling *Brn2* expression in post-mitotic neurons and may open the question as to whether *Sim1* and *Otp* act in parallel, or if one is downstream the other in controlling *Brn2* expression. Interestingly, in *Otp*^{-/-} embryos, *Sim1* expression is maintained in *lacZ*-positive cells in which *Brn2* is lost and, in *Sim1*^{-/-} embryos, *Otp* is expressed in the territory in which *Brn2* disappears. These findings provide strong in vivo evidence that *Otp* and *Sim1* act in parallel and are both required for proper expression of *Brn2* in the spv and its derivatives, the PVN and SON (Fig. 9C).

However, it is yet unclear whether *Otp*-mediated activation of TRH and SS also requires an additional intermediate transcription factor. We also show that in the ARN of *Otp*^{-/-} brains, SS, but not GHRH, expression is affected. Interestingly, in the ARN, the homeobox-containing gene *Gsh1* is essential for GHRH expression (Fig. 9C; Li et al. 1996). Therefore, summing up our data, *Otp* is required early for proper proliferating activity and, subsequently, for proper migration and terminal differentiation events that involve maintenance of *Brn2* expression, activation of parvocellular and magnocellular neuropeptide gene expression, axonal outgrowth, and cell survival (Fig. 9A–C). Finally, as no roles have been described so far for *Sim1*, *Brn2*, and *Gsh1* in modulating cell proliferation and/or migration of neuroendocrine precursor cells, *Otp* is the first example of a transcription factor required for controlling multiple developmental milestones that lead to the establishment of the main neuroendocrine cell lineages within the murine hypothalamus.

Materials and methods

Targeting vector, ES cell transfection, and selection of targeted clones

A 1.0-kb *DraI* fragment, located 60-bp upstream of the *Otp* translation start site and a 6.0-kb *EcoRI/XmnI* fragment spanning a region containing the second intron and the third exon,

were cloned in the pGN plasmid (Le Mouellic et al. 1990) and flanked the *lacZ* reporter gene and a neomycin resistance cassette, all in the same orientation. Fifteen micrograms of targeting vector were linearized by *KpnI* digestion and electroporated into 2×10^7 HM-1 ES cells. A total of 450 clones were analyzed by PCR and 3 homologous recombinants were identified with the following primers: sense primer, 5'-ACTTTGTTGTTGGC-TGGCTCTGA-3'; antisense primer, 5'-CAGTCACGACGTT-GTAAAACGACG-3' (arrows in Fig. 1A) and confirmed in Southern blot assays by hybridizing *ApaI* and *EcoRI*-digested genomic DNA with probes a and c (Fig. 1A).

Mouse production and genotyping

Two independent positive clones were injected into C57BL/6 blastocysts and the resulting chimaeric males back-crossed to B6/D2 F1 females. Genotyping was performed by PCR with two primers specific for the wild-type allele and located in the *Otp*-deleted sequence (sense primer, 5'-GAAAGATGCCGCC-GAGCTTCTG-3'; antisense primer, 5'-TGTCTGGGTCTTTG-GCGTCAC-3') (open arrowheads in Fig. 1A) and two primers specific for the *lacZ* gene (Acampora et al. 1995; filled arrowheads in Fig. 1A).

Western blot analysis

Total extracts were prepared from E13.5 embryos. A total of 80 µg of these extracts and 10 µg of nuclear extracts of HeLa cells transfected with plasmids overexpressing human *Otp* cDNA under a CMV enhancer-promoter (Simeone et al. 1994) were electrophoresed and transferred to nitrocellulose in a standard Western blot assay, probed with an affinity purified αOTP antiserum (Lin et al. 1999) (dilution 1:1000) and revealed by the standard Alkaline Phosphatase method.

β-Gal staining

Embryos were fixed in 4% paraformaldehyde for 15 min, followed by two washes with PBS for 10 min at room temperature. Staining was carried out overnight at 37°C in PBS containing 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, 2 mM MgCl₂, 20 mM Tris (pH 8), and 1 mg/ml X-Gal.

Histology and staining for calb-D28K

For histology, P1 brains and embryos were fixed in 4% paraformaldehyde for 16 hr, processed for wax embedding, sectioned at 7 µm and stained with Cresyl violet (brain). Calb-positive cells were revealed on brain and embryo sections by use of an anti-calb-D28K antibody (SWant) according to the manufacturer's instructions.

In situ hybridization

In situ hybridization experiments were performed as described (Simeone 1998). The *Dlx1* probe was the same as described previously (Simeone et al. 1994). The *Otp* and *lacZ* probes corresponded to probe b and c in Figure 1A. Probe b was a 500-bp long fragment spanning the *Otp* exon II; probe c is a 800-bp long fragment of the *lacZ* gene. *Sim1* and *Brn2* were two RT-PCR-amplified fragments spanning the region between amino acids 534 and 758 (for *Sim1*) and between amino acids 151 and 287 (for *Brn2*). The *Sim1* reporter (*Sim1 rep*) probe used in *Sim1*^{-/-} mutants was the same as described previously (Michaud et al. 1998).

BrdU labeling and detection of apoptotic cells

Pregnant mice at E10.3, E11.3, and E12.3 were injected intraperitoneally with BrdU solution (50 mg/kg body weight) and sacrificed after 1 hr, or at E12.5 and P1 (for mice injected at E10.3 and E11.3). Four embryos for each genotype for each BrdU administration were analyzed. Wax-embedded embryos were sectioned and adjacent sections were processed for BrdU detection or in situ hybridization. BrdU detection was performed according to Xuan et al. (1995).

To detect apoptotic cells, embryo sections were processed according to the TUNEL method (Garvieli et al. 1992). Four embryos were analyzed at E10.3, 11.3, 12.3, 14.5, and 15.5, and four brains at P1. Pregnant mice of two of the four embryos analyzed at E10.3, 11.3, and 12.3 were also injected with BrdU and processed in parallel for detection of both apoptotic and BrdU-positive cells.

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