Development 134, 4417-4426 (2007) doi:10.1242/dev.011262

Specification of hypothalamic neurons by dual regulation of the homeodomain protein Orthopedia

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In the developing hypothalamus, a variety of neurons are generated adjacent to each other in a highly coordinated, but poorly understood process. A critical question that remains unanswered is how coordinated development of multiple neuronal types is achieved in this relatively narrow anatomical region. We focus on dopaminergic (DA) and oxytocinergic (OT) neurons as a paradigm for development of two prominent hypothalamic cell types. We report that the development of DA and OT-like neurons in the zebrafish is orchestrated by two novel pathways that regulate the expression of the homeodomain-containing protein Orthopedia (Otp), a key determinant of hypothalamic neural differentiation. Genetic analysis showed that the G-protein-coupled receptor PAC1 and the zinc finger-containing transcription factor Fezl act upstream to Otp. In vivo and in vitro experiments demonstrated that Fezl and PAC1 regulate Otp at the transcriptional and the post-transcriptional levels, respectively. Our data reveal a new genetic network controlling the specification of hypothalamic neurons in vertebrates, and places Otp as a critical determinant underlying Fezl- and PAC1-mediated differentiation.

KEY WORDS: Neural development, Dopamine, Oxytocin

INTRODUCTION

The hypothalamus is a complex brain structure that regulates endocrine, behavioral and autonomic functions by means of its ability to affect both central and peripheral activities (Iversen et al., 2000). Accordingly, developmental impairments in hypothalamic differentiation are associated with defects in energy balance, and in neuroendocrine and psychiatric disorders (Swaab, 2004). For example, loss of hypocretin neurons leads to narcolepsy, and patients with Prader-Willi syndrome have a deficit in oxytocinergic (OT) neurons that is accompanied by hyperphagia and severe obesity (Peyron et al., 2000; Swaab et al., 1995). The medial area of the hypothalamus contains magnocellular and parvocellular neurons that control pituitary activities. The magnocellular neurons project to the posterior pituitary where they release oxytocin and arginine-vasopressin directly into the general circulation (Landgraf and Neumann, 2004). The parvocellular neurons affect the anterior pituitary by releasing hypophysiotropic hormones such as dopamine and somatostatin into the hypophysial-portal-vascular system (Markakis, 2002).

Hypothalamic development poses a challenging model for understanding neural patterning and specification because the hypothalamus contains multiple nuclei, each composed of several neuronal cell types that form connections with many parts of the nervous system (Markakis, 2002). Uncovering critical molecules regulating neural diversification of the hypothalamus is essential to understand how this elaborate brain region is formed. Insights into the differentiation of certain hypothalamic neurons has been contributed by targeted gene knockouts of the transcription regulators *Sim1*, *Brn2*, *Arnt2*, *Hmx2/3* and *Otp* (Acampora et al., 1999; Michaud et al., 2000; Michaud et al., 1998; Schonemann et al., 1995; Wang et al., 2004; Wang and Lufkin, 2000). The homeodomain-containing protein Orthopedia (Otp) is a key determinant controlling the specification of neuroendocrine hypothalamic neurons (Acampora et al., 1999; Wang and Lufkin, 2000). However, the signaling pathway(s) that regulate Otp and eventually lead to synchronized hypothalamic differentiation have not been elucidated.

Here, we focus on studying the development of dopaminergic (DA) and OT-like neurons [termed isotocinergic (IT) neurons] representing mammalian parvocellular and magnocellular cell types, respectively. We report the mode of regulation of zebrafish Otpb during the development of these two prominent neuronal clusters. We show that regulated expression of Otpb by two novel converging pathways coordinate the development of IT and DA neurons.

MATERIALS AND METHODS

Fish stocks

Fish breeding and maintenance were performed as previously described (Levkowitz et al., 2003). Experiments were performed in accordance with the Weizmann Institute IACUC protocol number 770104-1.

Plasmids and probes

Full-length *otpb* (see ZFIN ID: ZDB-GENE-990708-7 for nomenclature history), *pac1*, and *pacap1b* cDNAs were amplified by PCR from RNA that was isolated from embryos at 48 hours post fertilization. Identification of the *pac1* translation start site is detailed (see Fig. S8 in the supplementary material). Activated *pac1** (E239Q) was generated by PCR-based site-directed mutagenesis. To construct the Δ1-5-*pacap1b* mutant, site-directed mutagenesis was used to delete the cDNA nucleotides encoding amino acids 121-125 of the PACAP1b precursor protein. cDNA was subsequently subcloned into either the pCS2$^+$ plasmid or the heat-shock response element (HSE)-driven expression vector, pSGH2 (Bajoghli et al., 2004) and confirmed by nucleotide sequencing. Oligonucleotide primers that were used to amplify DNA templates for all digoxigenin (DIG)-labeled probe synthesis reactions are described in Table 1.

Immunostaining and in situ hybridization

Whole mount immunostaining with either polyclonal or monoclonal anti-tyrosine hydroxylase (TH) antibody (Chemicon, Temecula, CA) and in situ hybridization were performed as described (Levkowitz et al., 2003). Otp antibody was raised against a C-terminal Otp peptide and purified by affinity

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Accepted 19 September 2007

Table 1. Probes and real-time PCR primers used in this study

Probe size	Primers	GenBank accession no.	Gene
500 bp	Forward, CTCCGCAAGCTCTCGGTGTC Reverse, CTGCACTAATGTACAGTCAAGC	NM_178291	*isotocin* (*it*)
550 bp	Forward, GCAAAGAGAACTTACCCGACG Reverse, CAACATTCTTTCCGATGACAC	NM_183070	*somatostatin1* (*ss1*)
450 bp	Forward, AATGCGCCTCTGTCGACATTG Reverse, ACTGCTCACATCCTGTGGTACCG	AF025305	*hypocretin* (*hcrt*; also known as *orexin*)
1325 bp	Forward, GGGAGCTGCCCTGGATCCACT Reverse, TCAAACCACCAGCCAGTGATGGAG	AF318177	*dat*
722 bp	Forward, CACTACAAACCTCAAGTATTC Reverse, CCCACTTAACAATCATTG	NM_131100	*otpb*
307 bp	Forward, GACGTGCCGTTTCACCTC Reverse, CGAGTGCACCTTGTTTCT	XP_683186	*otpa*
1260 bp	Forward, ATGGCTTCCACAGTGAGAAAATGTC Reverse, CTGAAAAGACCCAAGACCGAGCTC	CR853299	*pac1*
400 bp	Forward, GAAGTCCTCCATCTCTTACGG Reverse, ATCTTTAGGACTGAATGTACAC	NM_214715	*pacap1b* (also known as *adcyap1b*)
65 bp	Forward, AATCGCGAGTGCAGGTCTG Reverse, GGTGGTCTTCTTGCGCTTCTT	XM_215445	*Otp* (rat)
70 bp	Forward, TGACCGAGCGTGGCTACA Reverse, CAGCTTCTCTTTAATGTCACGCA	NM_031144	*Actb* (rat)

chromatography as described (Lin et al., 1999). Following in situ hybridization, embryos were embedded into 1.5% agarose, dehydrated and embedded in paraffin. Paraffin blocks were sectioned (6 μm) on a microtome, mounted on slides and subjected to deparaffinization. Microwave-induced Otp antigen retrieval was performed in 10 mM citric acid (pH 6) for 10 minutes. Sections were blocked in PBS with 20% goat serum and 0.5% Triton X-100, and then incubated overnight at room temperature with affinity-purified rabbit Otp antisera (at 2 μg/ml). Sections were then washed with PBS and bathed with a goat anti-rabbit biotinylated antibody (at 1:200 dilution) for 1.5 hours at RT and visualized using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA).

Transient transfection, immunoblot and quantitative real-time PCR analysis

HEK293 cells were grown in 12-well plates and transfected (at 60% confluence) with a total amount of 1.0 μg/well of the indicated pCS2-based expression vectors using a standard calcium phosphate method. In some experiments, cells were incubated for the indicated time periods in the presence of different concentrations of a synthetic $PACAP_{38}$ peptide (Sigma-Aldrich, Rehovot, Israel). Proteins were harvested 24 hours post transfection in 150 μl of hot SDS sample buffer and 15 μl of the crude protein extract was fractionated by 10% SDS-PAGE followed by immunoblotting with an affinity-purified anti-Otp antibody. Thereafter, PVDF membranes were acid stripped and reprobed with a monoclonal anti-β-actin antibody (clone AC-74, Sigma-Aldrich, Rehovot, Israel).

PC12 cell line (clone number CRL-1721, 7-10 passage) was obtained from the ATCC Bioresource Center and propagated according to ATCC instructions. Nuclear-enriched protein extraction as well as total RNA preparation were previously described (Schreiber et al., 1989). For western blot analysis of Otp from either PC12 or zebrafish proteins, 20 μg total protein from each treatment was fractionated on 8.5% SDS-PAGE and immunoblotted with anti-Otp antibody as described above. Goat antisera directed against the nuclear regulator of chromosome condensation 1 (Rcc1; Santa-Cruz Biotechnology, Santa Cruz, CA) was used as an internal reference. Protein bands were quantified using an imaging densitometer and analyzed with Multi-Analyst Software (Bio-Rad Laboratories, Rishon Le Zion, Israel). [^{35}S]methionine incorporation and pulse-chase labeling procedures followed by anti-Otp immunoprecipitation and gel-autoradiography were performed according to published methods (Sambrook and Russell, 2001).

The level of endogenous *otp* RNA in $PACAP_{38}$-treated PC12 cells was determined by quantitative real-time PCR kit (DyNAmo HS SYBR Green qPCR Kit, Finnzymes, Finland) using 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA). Two micrograms of total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, USA) in a 20 μl reaction volume. PCR was performed in a 20 μl total reaction volume according to the kit instructions using rat-specific primers (Table 1) for either *otp* or *βactin*. Serial dilutions of the standard templates were also used for parallel amplification. The threshold cycles (Ct) were calculated and standard curves were plotted with Ct versus log template quantities. The quantities of samples were determined from the standard curves. *otp* levels were then normalized to those of *β-actin* in each corresponding sample.

Microinjection of morpholinos and RNAs

Capped RNAs were synthesized with mMESSAGE mMACHINE kit (Ambion, Austin, TX) from linearized $pCS2^{+}$ plasmids. The sequences of antisense morpholino oligonucleotides (Gene Tools, LLC, Corvallis, OR) targeted to *fezl*, *otpb* and *pac1* are listed in Table 2. Splice-blocking morpholinos to *fezl* (Exon2-Intron2), *otpa* (Exon2-Intron2) and *pacap1b* translation start blocking morpholino were previously described (Jeong et al., 2006; Ryu et al., 2007; Wu et al., 2006). *fezl otpb* and *pac1* RNAs were injected at the concentrations indicated in Table 3 into embryos at the two- to eight-cell stage. Injected embryos were allowed to develop at 28.5°C.

Table 2. Antisense morpholinos used in this study

Gene/MO name	Location	Sequence
fezl/sp1	Intron1-Exon2	AGACCTTTAAAAGAATAGAAACTGC
fezl/sp3	Exon2-Intron2	TATTTTAACCTACCTGTGTGTGAAT
otpb/sp2	Intron1-Exon2	TGCAGCATCCTTCATCCCTGTGGAC
otpa	Exon2-Intron2	ATCAGACTGCACCGCACTCACCTGC
pac1	ATG-Start	TCACTGTGGAAGCCATTTTGTCTGG
pacap1b	ATG-Start	GCCATGCTATTGCAGAGTAGGTAGA

Table 3. Concentration of morpholinos and RNAs used in this study

MO/RNA	Concentration (per embryo)
fezl-sp1 MO	3.0 ng/1.7 nl
fezl-sp3 MO	3.0 ng/1.7 nl
otpb-sp2 MO	4.0 ng/1.7 nl
otpa MO	4.0 ng/1.7 nl
pac1 MO	4.0 ng/1.7 nl
pacap1b MO	3.0 ng/1.7 nl
otpb RNA	15 pg/1.7 nl
pac1 RNA	200 pg/1.7 nl

Statistical analyses

Effects of the various genetic perturbations on DA and IT neurons were analyzed by counting the number of cells in the respective neuronal cluster of a given treated population of embryos and thereafter one-way ANOVA test was performed followed by the Tukey method for comparison of means using JMP software (SAS Institute, Cary, NC). The non-treated controls of the various treatments were pooled together after we determined (Tukey analysis) that there was no significant difference between the various control samples.

RESULTS

A common pathway controls the development of IT and DA neurons

To address the mechanism by which coordinated generation of different hypothalamic neurons is achieved, we focused on the DA and IT neuronal populations, which play major roles in neuroendocrine modulation of the anterior and posterior pituitary, respectively. IT neurons express isotocin-neurophysin (IT-NP), which is the zebrafish ortholog of the mammalian oxytocin-neurophysin (OT-NP) precursor protein (Unger and Glasgow, 2003). In wild-type (WT) embryos, IT neurons were readily detected in the neurosecretory preoptic nucleus (NPO), which is analogous to the mammalian supraoptic nucleus (SON) (Peter and Fryer, 1983), whereas hypothalamic DA cells developed in several clusters located at the basal plate posterior tuberculum (PT), adjacent to IT neurons (Fig. 1A). These DA clusters express the previously described (Puelles and Rubenstein, 2003; Rohr et al., 2001) hypothalamic markers *nk2.1a/titf1a*, *nk2.1b/titf1b* and *sim1* (N.B., H.N.-R. and G.L., unpublished material).

We have previously characterized the zebrafish mutant, *too few* (*tof*^{m808}), in which the development of hypothalamic DA neurons is significantly impaired because of a recessive mutation in the gene encoding the Fezl zinc-finger-containing protein (*fezl*, also known as *fezf2* – ZFIN) (Levkowitz et al., 2003). Simultaneous examination of IT and DA neurons revealed that the development of IT cells was attenuated in *tof*^{m808} embryos, which also displayed a clear deficit in DA neurons (Fig. 1B). However, hypothalamic hypocretin- and somatostatin-secreting neurons differentiated properly in the *tof*^{m808} mutant (Fig. 1C-F). Thus, proper development of both IT and DA neurons requires Fezl activity.

We sought to identify the molecular events underlying *fezl*/*tof*^{m808} activity. As IT and DA are generated in the NPO and PT, respectively, we performed an RNA in situ hybridization screen to identify candidate hypothalamic molecules that are expressed in these two adjacent hypothalamic nuclei (J.B. and G.L., unpublished material). We identified two genes, the homeodomain-containing gene, *otpb*, and the G-protein-coupled receptor, *pac1* (also known as *adcyap1r1* – ZFIN), that displayed a discernible expression pattern in the developing zebrafish hypothalamus (Figs 2, 3 and see Fig. S1 in the supplementary material). The zebrafish *otpb* gene is one of the two predicted orthologs of the mammalian *Otp*, and PAC1 is the predominant high-affinity receptor for the pituitary adenylate cyclase activating polypeptide (PACAP) neuropeptide (Vaudry et al., 2000). We observed that both Otp and PAC1 are expressed in the developing PT and NPO adjacent to or at the Fezl-expressing domains, respectively, suggesting that these molecules may be involved in differentiation of the two hypothalamic nuclei (Fig. 2A-D and see Fig. S1 in the supplementary material). Otp protein was detected later in terminally differentiated DA and IT neurons (Fig. 2E,F). Although PAC1 was readily detected in TH$^+$ DA neurons at 24-36 hpf, its expression in the later appearing (44-48 hpf) IT cells was nearly undetectable by the time these neurons underwent terminal differentiation (see Fig. S2 in the supplementary material and data not shown).

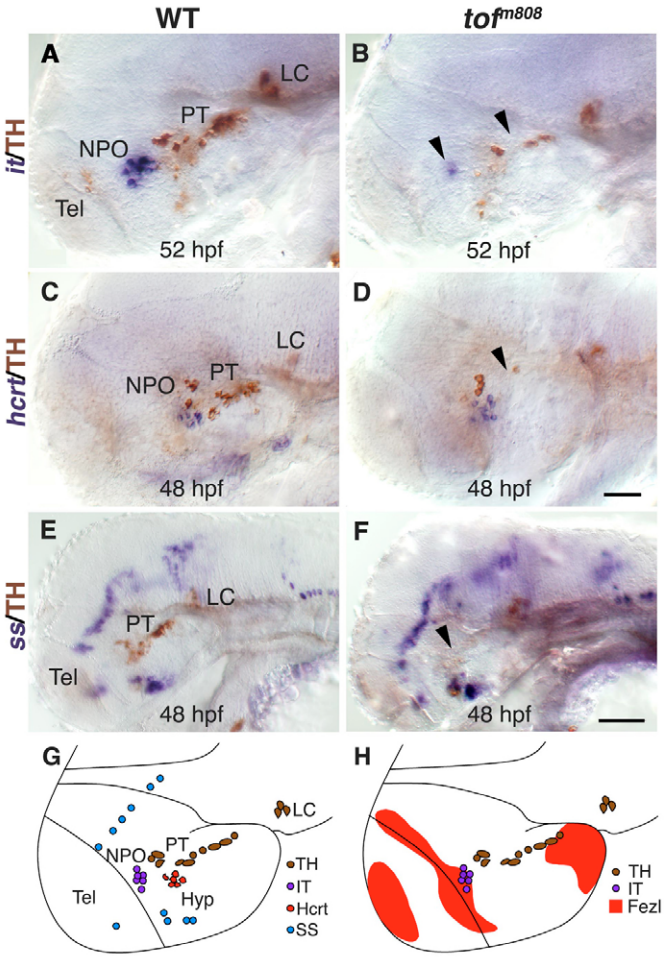

Fig. 1. Analysis of hypothalamic neurons in *fezl/tof*^{m808} mutant embryos. (**A-F**) Heterozygous (WT; A,C,E) embryos and their *too few*^{m808} (*tof*^{m808}) mutant siblings (B,D,F) were fixed at 52 (A,B), and 48 (C-F) hours post fertilization (hpf) and subjected to whole-mount in situ hybridization with antisense RNA probes directed against either the hypothalamic neuropeptide isotocin, which is the zebrafish ortholog of oxytocin (*it*; A,B), hypocretin/orexin (*hcrt*; C,D) or somatostatin (*ss*; E,F). After probe color development, all specimens were subjected to immunostaining with an anti-tyrosine hydroxylase (TH) antibody to detect DA neurons. WT heterozygous and *tof*^{m808} embryos were scored by TH staining followed by sequencing-based genotyping. Black arrowheads indicate deficiencies in DA and IT neurons in *too few* embryos. (**G**,**H**) Schematic representations of the examined hypothalamic cell types and of *fezl/tof* expression domains in a 2-day-old WT embryo. All panels show lateral views of the embryo, anterior to the left. Hyp, hypothalamus; LC, locus coeruleus; NPO, neurosecretory preoptic area; PT, posterior tuberculum; Tel, telencephalon. Scale bars: 50 μm in A-D; 100 μm in E,F.

fezl regulates *otpb*, but not *pac1* gene expression in the hypothalamus

As both Otp and Fezl are critical determinants of hypothalamic development we examined whether Fezl might regulate *otpb* by comparing its expression in WT embryos to *tof*^{m808} mutants and to

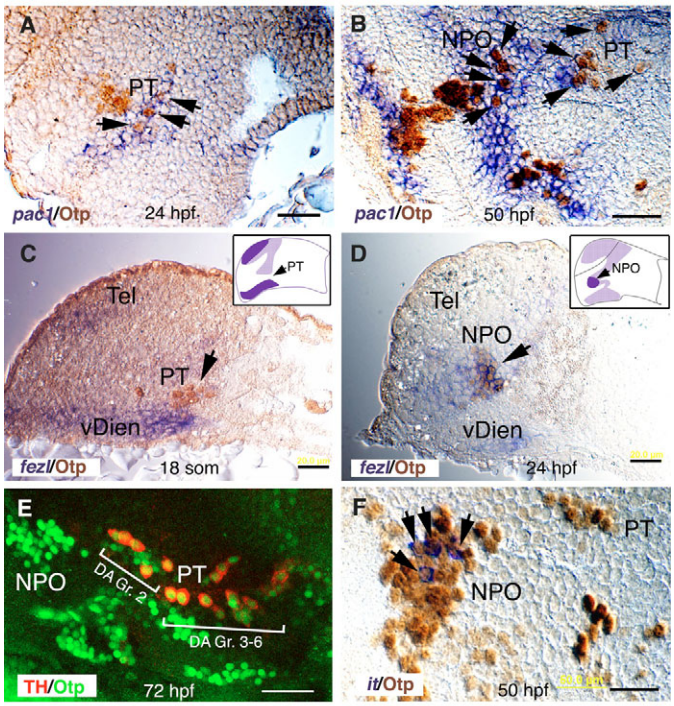

Fig. 2. Coordinated expression of *pac1, fezl* and Otp in the NPO and PT areas. (**A-D**,**F**) Sagittal paraffin sections (6 μm) through the NPO and PT (lateral view, anterior to the left). At the indicated stages of development, embryos were subjected to whole-mount in situ hybridization with probes directed against *pac1* (A,B), *fezl* (C,D) or isotocin (*it*; F). Thereafter, specimens were sectioned and immunostained with an anti-Otp antibody. Schemes of *fezl* expression domain (dark purple) at the plane of the section are shown in C and D. Labeled RNA probes were detected in the cytoplasm, whereas Otp antibody exclusively labeled the nuclei. (**E**) Projected confocal *Z*-stack images of embryos (lateral view, anterior to the left) that were subjected to double immunofluorescence staining with monoclonal and polyclonal antibodies directed against tyrosine hydroxylase (TH) and Otp, respectively. The two prominent clusters of dopaminergic neurons, group 2 (Gr. 2) and groups 3-6 (Gr. 3-6), are indicated. hpf, hours post fertilization; NPO, neurosecretory preoptic area; PT, posterior tuberculum; vDien, ventral diencephalon. Scale bars: 20 μm in A-D; 40 μm in E,F.

fezl knockdown embryos. *tof*^{m808} embryos displayed a complete loss of *otpb* gene expression in the PT area, and a slight reduction in *otpb*$^{+}$ cells of the NPO (Fig. 3B, *n*=70/70). The expression of *otpb* in the ventral diencephalon and hindbrain was unaltered in *tof*^{m808} mutants. Complete inactivation of Fezl by injecting two independent splice-blocking morpholino oligonucleotides into *tof*^{m808} (denoted *tof*^{fezlMO}) resulted in a stronger effect; markedly diminished *otpb* expression was observed in the PT, NPO and ventral diencephalon, but not in the hindbrain (Fig. 3C, *n*=10/15). The latter result suggests that the *tof*^{m808} allele of *fezl* is a hypomorph that retains residual transcriptional activity. In agreement with the stronger effect of *fezl* knockdown on *otpb* expression, the *tof*^{m808} mutant allele displayed a delay in IT development whereas *tof*^{fezlMO} embryos displayed a sustained loss of IT neurons (Fig. 3G). Finally, the expression of the G-protein-coupled receptor *pac1* in the PT, NPO and pituitary was not affected by gene perturbations of *fezl*/*tof* (Fig. 3D-F, *n*=60). Hence, the Fezl transcription factor specifically regulated the expression of *otpb* at two distinct hypothalamic nuclei that produce IT and DA neurons.

otpb and *pac1* control the differentiation of IT and DA neurons

To address the role of *otpb* in DA and IT development we examined the phenotype of these neurons following genetic perturbation of *otpb*. Consistently with the regulated expression of *otpb* in the NPO and PT, injection of a splice-blocking morpholino directed against *otpb* (denoted *otpb*MO) impaired the development of IT and DA neurons (Fig. 4B,C and see Fig. S3 in the supplementary material). We then analyzed the effect of *otpb*MO on discrete DA clusters of the PT in 2- to 3-day-old embryos. These neuronal clusters can be readily identified in the PT of 2- to 5-day-old zebrafish embryos (Rink and Wullimann, 2002). Interestingly, *otpb*MO treatment had a stronger effect on the posterior hypothalamic DA groups 3-6, whereas group 2 was less affected (Fig. 4C and see Fig. S3 in the supplementary material).

The G-protein-coupled receptor, *pac1*, was detected in the zebrafish NPO and PT before and during the period of DA and IT differentiation (Figs 2 and 3 and see Fig. S2 in the supplementary material). This suggested that *pac1* might be a good candidate transducer of an extracellular cue, which may be involved in DA and IT development. Indeed, knockdown of *pac1* gene activity impaired the development of both IT and DA neurons (Fig. 4F and see Fig. S3 in the supplementary material). As in the case of *otpb*MO, inactivation of *pac1* mainly affected hypothalamic DA groups 3-6, implying a common genetic pathway shared by *otp* and *pac1* (Fig. 4B,C,F). Similarly to the reported *tof*^{m808} phenotype (Levkowitz et al., 2003; Rink and Guo, 2004), DA cell groups in the retina, telencephalon and pretectal diencephalon, as well as TH^{+};Otp^{+} noradrenergic neurons of the locus coeruleus (LC), were not affected by *otpb* or *pac1* inactivation (data not shown). Consistently with the *pac1* knockdown phenotype, the high-affinity ligand to PAC1, PACAP1b, was expressed in the NPO and PT, and was found to be necessary for IT and DA development (see Figs S1,S3 in the supplementary material). The *pacap1b* morphant, however, had a more pleiotropic effect including diminution of mid-hindbrain boundary and cerebellar structures, suggesting that PACAP1b may bind to additional G-protein-coupled receptors other than PAC1 (Vaudry et al., 2000).

pac1, otp and *fezl* form a genetic network controlling hypothalamic differentiation

Our results thus far suggest that differentiation of IT neurons as well as of discrete clusters of hypothalamic DA cells is regulated by the transcription factors Fezl and Otp and by the G-protein-coupled receptor, PAC1. We next performed a genetic epistasis analysis to reveal the hierarchical interaction between *fezl, otpb* and *pac1*. For gain of function of Otpb we first determined the dose of *otpb* RNA (7-15pg) that could rescue the *otpb* morphant without affecting patterning (Fig. 4D and data not shown). In order to activate the PAC1-mediated signaling pathway, we generated a constitutively active form of the PAC1 receptor (Cao et al., 2000), denoted PAC1*. This construct had no obvious effect on patterning even at the highest dose used (Fig. 4H and data not shown). Injection of *pac1** mRNA rescued the *pac*1 knockdown phenotype, but could not complement *otpb* deficiencies, suggesting that Otp is acting downstream of PAC1 (Fig. 4E,H). Consistently, the deficiencies in DA and IT neurons that were caused by injection of *pac1*MO could be rescued by co-injection of mRNA encoding to the Otpb protein (Fig. 4G). Rescue of *pac1* morphant by *otpb* mRNA was not due to upregulation of endogenous PAC1 or its ligand PACAP1b (see Fig. S5 in the supplementary material).

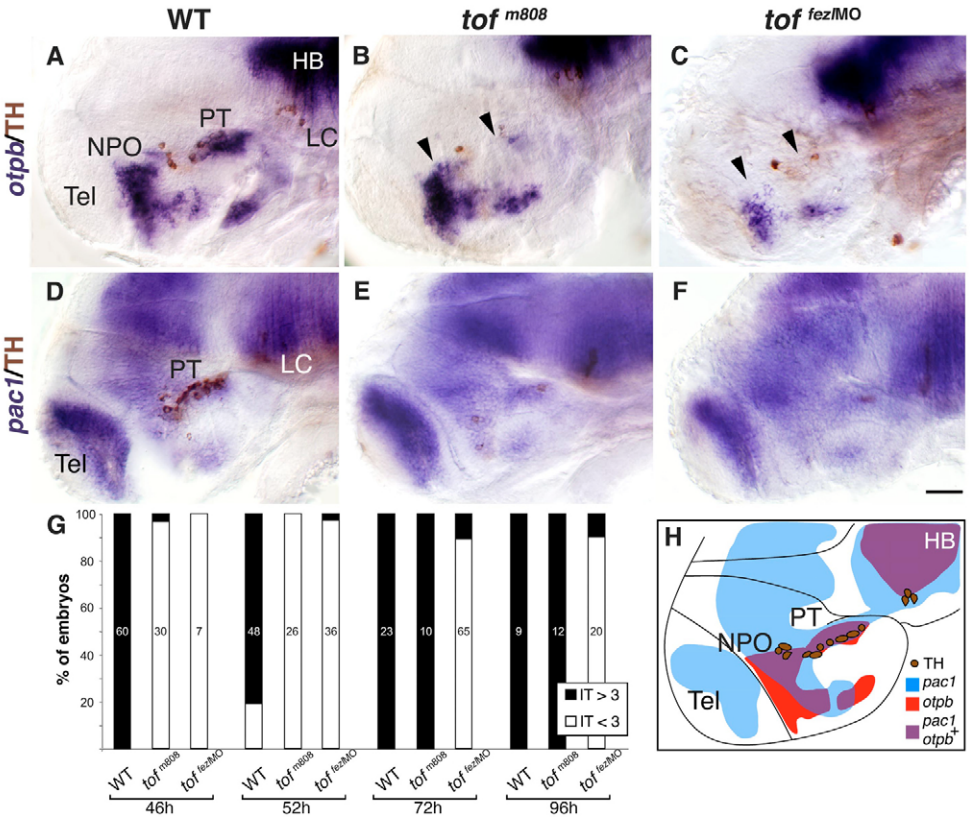

Fig. 3. Aberrant *otpb* expression in *fezl*-deficient embryos. Embryos (at 52 hpf, lateral view) were subjected to whole mount in situ hybridization with either antisense *otpb* (**A-C**) or *pac1* (**D-F**) RNA probes followed by immunostaining with an antibody directed against tyrosine hydroxylase (TH; A-F) that detects DA neurons. (A,D) Wild type (WT). (B,E) *tof*^{m808} mutants. (C,F) *tof*^{m808} embryos were injected, at one-cell stage, with an antisense Fezl morpholino (*fezl*MO) that blocks proper splicing leading to retention of intron 2 and a shorter protein that lacks most of the zinc finger domain (sp3; see Fig. S6 in the supplementary material). WT heterozygous and *tof*^{m808} embryos were scored by TH staining followed by sequencing. Black arrowheads mark deficient *otpb* expression domains. (**G**) Delayed IT development in *tof*^{m808}. Relative number of IT/OT neurons in *too few* (*tof*^{m808}) embryos, *tof*^{fezlMO} and their WT siblings at different developmental stages. *tof*^{fezlMO} indicate embryos, in which *fezl*-directed antisense morpholino was injected into *tof*^{m808}. Embryos were scored by co-staining of IT and TH followed by sequencing of the *tof*^{m808} mutation. WT embryos display 4-7 IT cells (IT>3) on each side of the brain whereas *tof*^{m808} display a deficit (IT<3) in IT cell number between 46 and 52 hours of development. The bars were normalized to 100%. The number of scored embryos is indicated on each bar. (**H**) A scheme depicting an overlay of TH, *otpb* and *pac1* expression domains as they appear in WT embryos (at 52 hpf). HB, hindbrain; LC, locus coeruleus; NPO, neurosecretory preoptic area; PT, posterior tuberculum; Tel, telencephalon. Scale bar: 50 μm.

Although *otpb* expression was diminished in the DA-deficient *tof*^{m808} mutant (Fig. 3), overexpression of *otpb* RNA could not rescue the *tof* phenotype (data not shown). We hypothesized that this was due to insufficient Otpb protein expression levels in hypothalamic precursors. We thus attempted to rescue *tof*^{m808} by injection of heat-shock- inducible expression vector, which drives either Otpb or PAC1* together with a GFP tracer (Bajoghli et al., 2004), followed by selection of mosaic embryos expressing high GFP levels in the hypothalamus (see Fig. 5F). Using this system, we were able to obtain complete rescue of the *tof* phenotype by gain of function of Otpb (Fig. 5C; *n*=9/11), but not of PAC1* (Fig. 5D; *n*=0/12). Taken together the above epistatic analyses show that *otpb* acts downstream of both *fezl* and *pac1* and that proper development of DA and IT neurons requires the activation of two signaling pathways that converge to coordinate *otpb* function at different hypothalamic nuclei.

To determine the critical period of competence, in which Otpb is able to control DA development we temporally expressed *otpb* in zebrafish embryos using the aforementioned heat-shock-inducible Otpb construct. Induction of ectopic expression of the Otpb protein was detected at 90 minutes after shifting the temperature from 28°C to 38°C (data not shown). Gain of function of Otpb by such temporal temperature shift resulted in a supernumerary DA phenotype (~twofold increase) when Otpb was induced at 7 and 10 but not at 14 hpf (Fig. 5E-G). Accordingly, Otpb could rescue the *tof* phenotype when its expression was induced at 7 but not 14 hpf (Fig. 5C and data not shown). As the majority of DA precursors exit the cell cycle by 14-16 hpf (N. Russek-Blum and G.L., unpublished results) we conclude that Otpb is required in hypothalamic DA progenitors and not in post-mitotic neurons.

PAC1 and its ligand PACAP regulate the levels of Otp protein

Although we demonstrated that *otpb* is epistatic to *pac1*, knockdowns of *pac1* and of *pacap1b* had no effect on *otpb* transcript levels (Fig. 6C and supplementary material Fig. S4). Therefore, unlike the evident effect of *fezl*/*tof* on the levels of *otpb* RNA (Fig. 3A-C), regulation of *otpb* transcription could not account for the genetic interaction between *pac1* and *otpb*. We then monitored Otp protein in whole embryos following knockdown of either *pac1* or its ligand, *pacap1b* (Fig. 6 and see Fig. S4 in the supplementary material). The levels of Otp protein were moderately reduced in the hypothalamus and hindbrain following injection of *pac1*MO (Fig. 6D; *n*=25/30). Western blot analysis of Otp, which allows more accurate quantification of the effects of *pac1*MO, resulted in a 50% decrease in total Otp protein levels (Fig. 6D). As the anti-Otp antibody we used was raised against a C-terminal epitope of Otp (see Materials and methods) it recognizes both Otpb and its paralog Otpa. These two genes have nearly complete overlapping expression domains (Ryu et al., 2007) (data not shown). To demonstrate the net effect of PAC1 on Otpb protein we compared Otp immunoreactivity in *otpa*

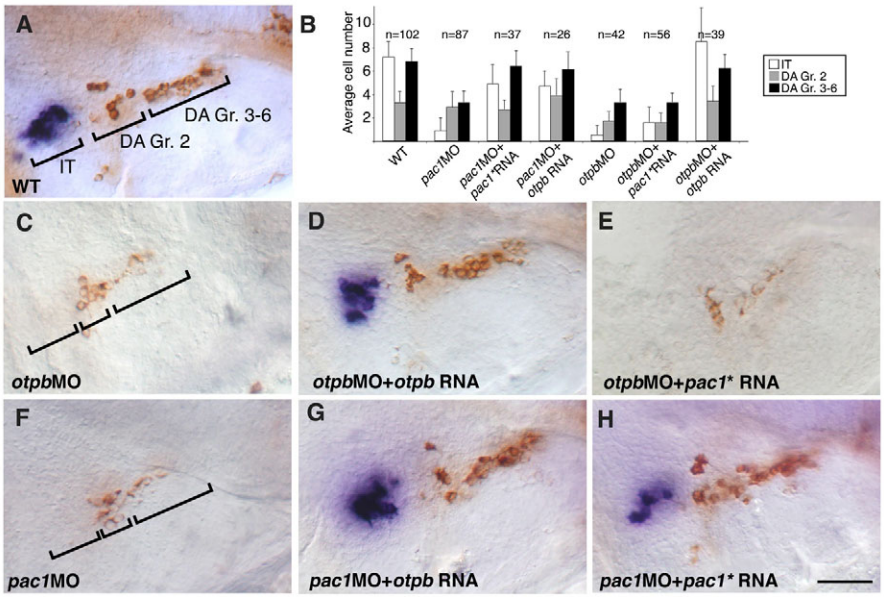

Fig. 4. Genetic interactions between *pac1* and *otpb*. (**A**,**C-H**) High-resolution micrographs of embryos (at 52 hpf, lateral view, anterior to the left) that were subjected to in situ hybridization with an isotocin (IT)-directed probe, followed by immunostaining with an anti-tyrosine hydroxylase (TH) antibody. The two prominent clusters of dopaminergic (DA) neurons (Gr. 2 and Gr. 3-6), stained in brown, as well as isotocinergic (IT) neurons, stained in purple, are indicated. (**B**) Bar chart showing average cell counts of isotocinergic (IT), dopaminergic Group 2 (DA Gr. 2) and Groups 3-6 (DA Gr. 3-6). Error bars indicate s.d. The number of embryos (*n*) is shown above. In order to represent unilateral cell number (as shown in the micrographs), neurons were counted on both sides of the brain and the total number was divided by two. Statistical analysis for all treatments was done by ANOVA test. Wild-type (WT) embryos were injected with antisense morpholinos directed against either *otpb* (*otpb*MO; C,D,E and see Fig. S7 in the supplementary material) or *pac1* (*pac1*MO; F,G,H). (C-H) Embryos were injected with in vitro synthesized capped mRNA encoding either Otpb (D,G; at 7-15 pg per embryo) or a mutant form of PAC1 (denoted *pac1**) containing a single E239Q base substitution that renders it constitutively active (E,H; at 200pg per embryo). Injected *pac1** and *otpb* RNAs were constructed without their respective morpholino-binding site, thus enabling a genuine gene-function complementation rather than competition for morpholino binding. The amount of injected mRNA in Otpb and PAC1 gain-of-function experiments was determined by titrating the minimal doses of mRNA that could rescue *otpb* and *pac1* morphant phenotype, respectively. Embryos that showed abnormal brain morphology were omitted from our analysis. Scale bar: 50 μm.

morphants (Fig. 6F; *n*=22) versus *pac1*+*otpa* double morphants (Fig. 6H). Otp immunoreactivity was markedly reduced in the double morphant with no significant change in the levels and in expression pattern of *otpb* RNA, suggesting that PAC1 might modulate *otpb* post-transcriptionally (Fig. 6G,H; *n*=29/30). To further examine this possibility we expressed zebrafish PAC1, PACAP1b and Otpb in a heterologous cell culture system. In agreement with Lin et al. (Lin et al., 1999), transient expression of zebrafish Otpb in the human HEK293 cell line followed by western blot analysis detected an Otp-immunoreactive protein band with an apparent molecular mass of 50 kDa (see Fig. S4 in the supplementary material). Otpb protein levels were increased two- to threefold after coexpression of Otpb with PAC1*, the constitutively activated form of the receptor (Fig. 7A). Higher induction (sixfold) of Otpb protein was detected following coexpression of Otpb and PAC1 together with the PACAP1b precursor protein, but not with an N-terminally truncated form of PACAP1b, denoted Δ1-5-PACAP, which acts as a PAC1 antagonist (Robberecht et al., 1992) (Fig. 7A). Hence, the in vivo genetic interaction between zebrafish *pac1* and *otpb* could be reconstituted in a mammalian system in vitro.

We next examined whether stimulation of PAC1 with a synthetic ligand could affect Otp protein levels. Treatment of *otpb*;*pac1* double-transfected cells with increasing concentrations of a synthetic $PACAP_{38}$ peptide led to a four- to fivefold increase of Otpb protein (Fig. 7B). We then tested the effects of $PACAP_{38}$ on *otp* gene products in the rat PC12 cells, which express endogenous Otp (Fig. 7C,D), and in which the PACAP-PAC1 pathway has been extensively studied (Vaudry et al., 2000). Treatment of PC12 cells for up to 120 minutes with different concentrations of a synthetic $PACAP_{38}$ neuropeptide led to a fivefold increase of endogenous levels of Otp protein (Fig. 7C,E). In accordance with our in vivo results, quantitative real-time PCR analysis indicated that the level of *otp* RNA in PC12 cells was unaffected by PACAP stimulation, suggesting that a biochemical pathway triggered by PACAP and its receptor PAC1 regulates Otp at the post-transcriptional level (Fig. 7E). To determine the nature of this post-transcriptional control we examined the ability of PACAP to affect Otp protein stability and synthesis. Otp stability was examined by treating PC12 cells with the translation inhibitor cycloheximide (CHX) and thereafter monitoring the levels of Otp in the absence and presence of PACAP. This analysis showed that Otp protein was relatively stable throughout the time of the experiment and that PACAP-induced accumulation of Otp was blocked by CHX (Fig. 7D,E). This result was corroborated by [^{35}S]methionine pulse-chase kinetics analysis of Otp protein (see Fig. S9 in the supplementary material). To examine Otp synthesis, we measured [^{35}S]methionine incorporation into Otp in the presence or absence of PACAP. The rate of CHX-sensitive [^{35}S]methionine incorporation into Otp was significantly increased upon application of PACAP to PC12 cells (Fig. 7F). Taken together the above results indicate that PACAP controls the levels of Otp by promoting Otp synthesis without affecting the stability of both protein and mRNA.

In conclusion, we identified a novel regulatory network of cell-intrinsic and cell-extrinsic cues that act together to maintain coordinated development of hypothalamic DA and OT-like neurons.

DISCUSSION

In this study, we examined the genetic and biochemical basis for the apparent coordinated fashion in which DA and IT/OT neurons are generated within the relatively small hypothalamic territory. We show that the integration of transcriptional and post-transcriptional inputs that modulate the levels of the homeodomain-containing protein Otp in distinct hypothalamic nuclei (i.e. PT and NPO) may trigger specific differentiation programs that promote DA and IT identities in time and space (Fig. 8).

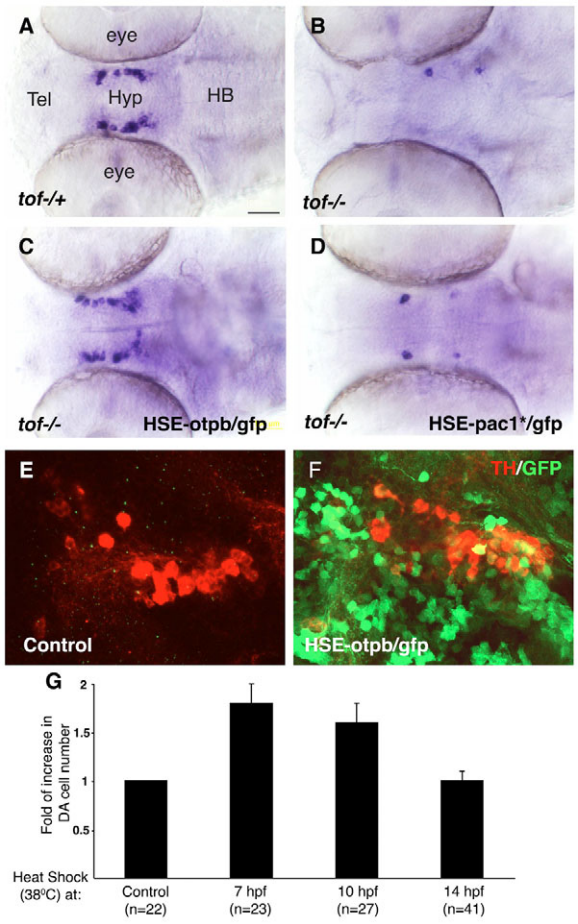

Fig. 5. Otpb is a critical downstream effector of Fezl in DA progenitors. (**A-F**) Embryos were injected with either a buffer solution (A,B,E) or DNA constructs containing a heat shock element (HSE) expression cassette that drives a GFP tracer together with either *otpb* (C,F; at 10-20 pg per embryo) or the constitutively active *pac1** (D) cDNAs and were grown at 28°C. At 7 hours post fertilization (hpf) embryos were transferred to a permissive temperature of 38°C for a period of 45 minutes and were shifted back to a 28°C incubator. Mosaic embryos expressing the GFP tracer in the ventral diencephalon were selected for further analysis and were thereafter harvested at 52 hpf. DA neurons were scored by either an RNA probe directed to dopamine transporter (*dat*; A-D) or an anti-tyrosine hydroxylase (TH) antibody (E,F). A-D are siblings derived from a cross between *tof*$^{m808-/-}$ and *tof*$^{m808-/+}$ fish. Subsequent to the phenotypic analysis, embryos were separated into a multiwell dish and were genotyped by sequencing. (**G**) Bar chart showing the relative changes in DA cell number following conditional activation of Otpb in WT embryos that were injected with a plasmid harboring a heat shock element-driven *otpb/gfp* (HSE-*otpb/gfp*) expression cassette. At the indicated times after fertilization, embryos were subjected to a 45 minute temperature shock (38°C) and the number of *dat*$^{+}$ hypothalamic DA neurons in each embryo was scored at 52 hpf. Scale bar: 50 μm.

Tight regulation of Otp during hypothalamic development

It has been suggested that the intersection between the secreted molecules Shh, Fgf8 and Bmp7 creates an induction site for hypothalamic DA identity (Ohyama et al., 2005; Ye et al., 1998). In spite of these reports, the regulation of cell-autonomous determinants, which presumably convert patterning signals into

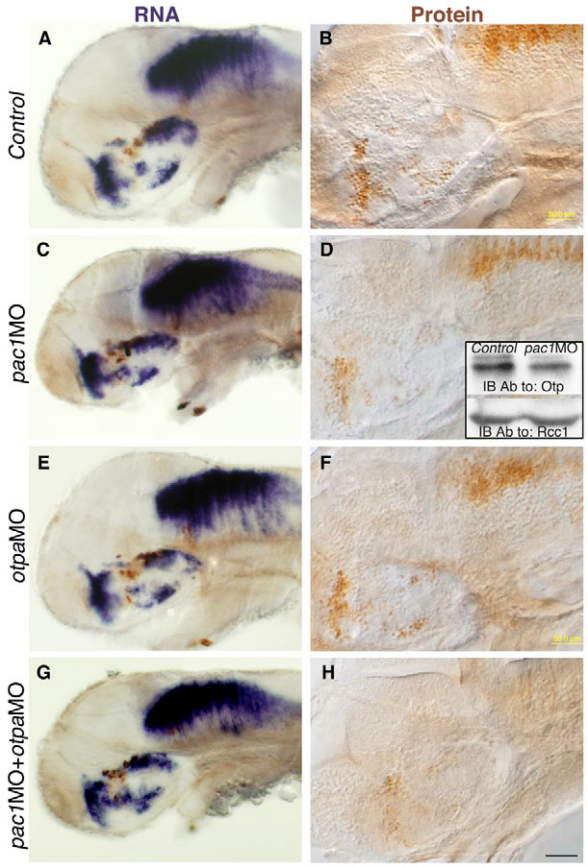

Fig. 6. Regulation of the Otp protein by PACAP/PAC1 signaling pathway. (**A-H**) Embryos were injected with either a control *pac1* missense morpholino (*Control*, A,B), or with antisense morpholinos directed against *pac1* (*pac1*MO, C,D), *otpa* (*otpa*MO, E,F) or combination of *pac1*+*otpa* (*pac1*MO+*otpa*MO, G,H) and were thereafter (52 hpf) subjected to either in situ hybridization with an *otpb*-directed probe (A,C,E,G), or to immunohistochemistry with an anti-Otp antibody (B,D,F,H). Inset in panel D shows western blot analysis of nuclear-enriched protein extracts from control and *pac1*MO-injected embryos using antibodies directed to either Otp or the nuclear protein Rcc1 (internal protein loading control). The activity of each morpholino was evaluated by anti-tyrosine hydroxylase (TH) immunostaining. Scale bars: 100 μm in A,C,E,F; 50 μm in B,D,F,H.

precise control of hypothalamic development, is poorly understood. Otp is a critical cell-intrinsic determinant, which controls the fates, migration and terminal differentiation of mammalian hypothalamic neuroendocrine cells (Acampora et al., 1999; Wang and Lufkin, 2000). We show that regulation of *otp* levels is important for the spatial and temporal development of IT and DA neurons. We suggest that the tight regulation of Otp is achieved by two sequential manners: first transcription of *otp* mRNA is induced by Fezl, then Otp protein levels are modulated by PAC1 (Fig. 8). In support of this model, we show that the transcript levels of *otpb* were markedly affected in the absence of *fezl/tof* gene function and that the levels of Otp protein were controlled by PAC1 and its ligand PACAP (Figs 3 and 6). Our in vitro analyses show that PACAP affects the rate of Otp protein synthesis, providing a mechanism for the post-transcriptional control of *otp*, which was observed in vivo (Fig. 7). Interestingly, PACAP exerts a persistent post-transcriptional effect on the steady-state levels of tyrosine hydroxylase in PC12 cells (Corbitt et al., 1998).

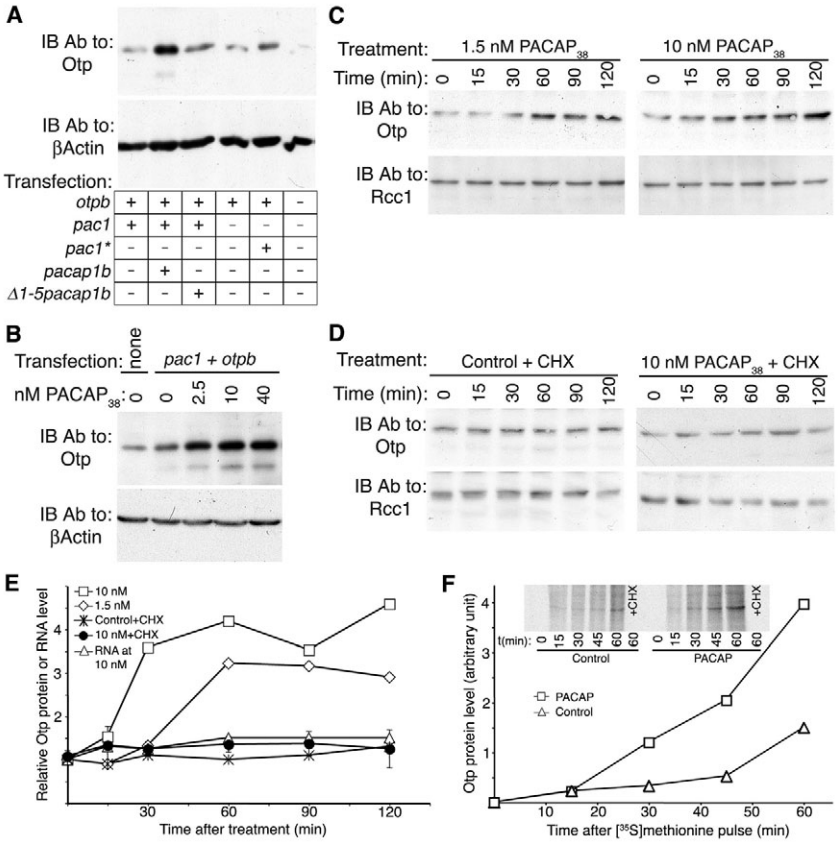

Fig. 7. PACAP regulates Otp post-transcriptionally. (**A**,**B**) Western blot of equal amounts of protein extracts from HEK293 cells with an anti-Otp antibody followed by an anti-β-actin antibody that served as an internal reference. (A) HEK293 cells were transiently transfected with different combinations of zebrafish cDNAs as indicated and harvested 24 hours post transfection. *pac1** encoded a constitutively activated form of *pac1*. The full-length form of zebrafish *pacap1b* precursor gene, gives rise to several proteolytically cleaved neuropeptides including growth hormone releasing hormone (GHRH) and PACAP1b (Wang et al., 2003). *Δ1-5pacap1b* cDNA had an internal deletion of the first five amino acids of the mature PACAP peptide. (B) HEK293 cells were transfected with the indicated zebrafish cDNAs and 24 hours later monolayer cultures were treated with different concentration of a synthetic $PACAP_{38}$ peptide for a period of 120 minutes. (**C**,**D**) Western blot of nuclear-enriched protein extracts from PC12 cells using either an anti-Otp or anti-Rcc1 antibody (internal control for a ubiquitous nuclear protein). Cells were treated with different concentrations of either $PACAP_{38}$ peptide alone (C), 50 μg/ml cycloheximide (Control+CHX) or $PACAP_{38}$+CHX (D) and harvested at the indicated time points. (**E**) Standardized Otp protein and RNA (n=6) levels as a function of time after $PACAP_{38}$ application to PC12 cells. *otp* RNA levels were determined by quantitative real-time PCR (RNA at 10 nM). (**F**) Kinetics of newly synthesized Otp protein in the absence (Control) or presence of 10nM PACAP. PC12 cells were incubated with methionine-free medium containing [^{35}S]methionine (at 100 μCi/ml). Protein extracts were made at the indicated times and equal amounts of total protein were subjected to immunoprecipitation with an anti-Otp antibody followed by gel electrophoresis and autoradiography (inset). The correct position of the Otp band was confirmed by performing immunoprecipitation and western blot on the same extracts and by Otp peptide displacement. Ab, antibody; CHX, cycloheximide; IB, immunoblot.

Intrinsic and extrinsic control of DA and IT differentiation

fezl, *otp* and *pac1* are coordinately expressed in the developing hypothalamus (Fig. 2). We report here that all three molecules are necessary for proper development of hypothalamic DA and IT neurons (Figs 4 and 5). Our epistatic and gene expression analyses are consistent with two parallel pathways that co-regulate IT and DA development by acting upstream of *otpb*.

The levels of *otpb* expression may also determine the time of appearence of IT neurons. Thus, the expression of *otpb* in the NPO was strongly affected in a null tof^{fezlMO} mutant (i.e. tof^{m808} injected with *fezl*MO) and only mildly affected in the tof^{m808} hypomorph (Fig. 3A-C). These levels of *otpb* transcripts correlated with the sustained absence of IT in tof^{fezlMO} and delayed IT development in the tof^{m808} hypomorph allele (Fig. 3G). Conversely, the deficit in DA neurons in tof^{m808} is maintained throughout embryogenesis and in adult mutant animals (Rink and Guo, 2004). The varied sensitivities of *otp*, IT and DA to Fezl activity correlate with *fezl* and *otp* expression patterns: whereas *fezl* and Otp colocalize in the NPO where IT cells develop, only *otp* is found in DA neurons of the PT (Fig. 2). Lack of Fezl-Otp colocalization in the PT is consistent with our previously published mosaic analysis showing that *fezl*/*tof* regulates hypothalamic DA development in a non cell-autonomous manner (Levkowitz et al., 2003). Hence, coordination of IT and DA cells may be attained by different sensitivities of the *otpb* promoter to Fezl-mediated signal at different hypothalamic territories.

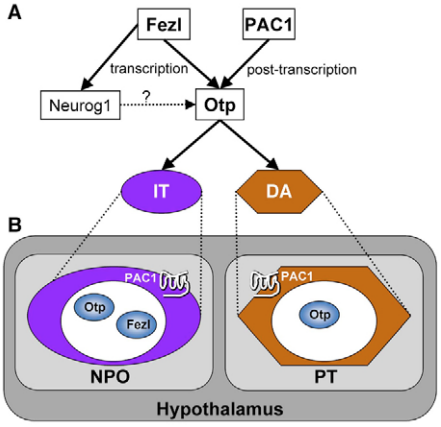

Fig. 8. A model for the development of IT and DA neurons. (**A**) Regulatory network underlying isotocinergic (IT, purple oval) and dopaminergic (DA, brown hexagon) neurons in the zebrafish hypothalamus (see text). Regulation of Otp by the proneural gene product Neurogenin1 (Neurog1) are yet to be determined (dashed arrow, see Discussion). (**B**) Expression of *pac1*, *fezl* and *otp* in either IT neurons that reside in the neurosecretory preoptic nucleus (NPO) or DA neurons that reside in the posterior tuberculum (PT) are schematically shown.

Control of neuronal specification processes by Fezl

Recent studies described the phenotype of Fezl-deficient mice. Similarly to zebrafish, mammalian Fezl is expressed in the telencephalon and diencephalon (Hirata et al., 2004; Mutsuga et al., 2005). In the mouse telencephalon, Fezl is required for fate specification and axonal projections of cortico-spinal motor neurons, subplate cortical neurons and deep-layer pyramidal neurons (Chen et al., 2005a; Chen et al., 2005b; Hirata et al., 2004; Molyneaux et al., 2005). A close homolog of Fezl, denoted Fez, is expressed in the mouse hypothalamus in partially overlapping domains with Fezl and *fez-fezl* double deficient mouse displays defects in diencephalic subdivisions (Hirata et al., 2006a; Hirata et al., 2006b). A similar role for zebrafish Fezl in diencephalic patterning was also reported (Jeong et al., 2007).

The precise regulation of Otp, DA and IT by the *tof*^{m808} hypomorph (Figs 1 and 3) allows us to separate the role of Fezl in regional patterning from its more selective role in cell specification. The fact that Otpb could rescue the *fezl/tof* phenotype indicates that Otpb is a critical target of Fezl that underlies its effect on hypothalamic differentiation. In this respect, the proneural gene *neurogenin1* (*neurog1*) is regulated by Fezl and is both necessary and sufficient for zebrafish DA development (Jeong et al., 2006). Similarly, *dlx2*, which controls specification of ventral thalamic DA progenitors in the mouse, is regulated by Fezl (Andrews et al., 2003; Yang et al., 2001). Neither *neurog1* nor *dlx2* is affected in either *otpb* or *pac1* morphants (data not shown), suggesting that Fezl may control both early regional diencephalic commitment, which is mediated by Neurog1 and Dlx2, and later cell-type specification, which is mediated by Otp (Fig. 8). Notably, there are two zebrafish orthologs of the mammalian *Otp* gene (denoted *otpa* and *otpb*). Although this study demonstrates the function and mode of regulation of *otpb*, a similar deficit in diencephalic DA neurons was recently found in a null mutant allele of *otpa*, suggesting that the activity of both *otpa* and *otpb* is required for hypothalamic development (Ryu et al., 2007). We found that the transcription of *otpa* was affected in the absence of the *fezl/tof* gene, reinforcing the significance of Fezl in regulating hypothalamic cell fate decisions (see Fig. S5 in the supplementary material).

Although the development of diencephalic neurons was not analyzed in detail, hypothalamic neurons appear to be present in Fezl$^{-/-}$ mice (Chen et al., 2005a; Hirata et al., 2004). However, in *fezl/tof*^{m808} mutants, *otpb* morphants and in *otp*-deficient mice, selective groups of hypothalamic DA neurons are reduced or missing, whereas other DA groups develop normally (Rink and Guo, 2004; Ryu et al., 2007) (Fig. 4). Further analysis is necessary to clarify whether subsets of hypothalamic neurons are affected in *fez/fezl*-deficient mice.

Finally, activation of the Otp regulatory network might be relevant to adult physiological states as *fezl*, *pac1* and oxytocin are upregulated in the rat SON in response to hyper-osmotic conditions and *fezl* and oxytocin are downregulated following sustained hypo-osmolality (Gillard et al., 2006; Mutsuga et al., 2005). Moreover, PACAP is enriched in rat mesencephalic DA neurons and protects DA neurons from neurotoxin-induced death (Chung et al., 2005; Grimm et al., 2004; Reglodi et al., 2004).

In sum, our data reveal two novel genetic pathways, which control Otp activity during differentiation of hypothalamic DA and OT-like neurons and may be relevant to hypothalamic developmental defects that cause metabolic and psychiatric clinical disorders.

Thanks are due to Thomas Czerny for kindly providing the pSGH2 expression vector; E. Peles, O. Reiner, M. Fainzilber and T. Volk for comments on this work; Shifra Ben-Or and Amos Gutnick for help with the bioinformatics and statistical analyses; Philippe Vernier and members of the Levkowitz lab for stimulating discussions. This study was supported by the Israel Science Foundation grant number 944/04, Parkinson Disease Foundation, Benozyio Center for Neurological Disorders, The Helen and Martin Kimmel Stem Cell Research Institute and Minna James Heineman Foundation. G.L. is an incumbent of the Tauro Career Development Chair in Biomedical Research.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/24/4417/DC1

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