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Canonical Wnt signaling through Lef1 is required for hypothalamic neurogenesis

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Although the functional importance of the hypothalamus has been demonstrated throughout vertebrates, the mechanisms controlling neurogenesis in this forebrain structure are poorly understood. We report that canonical Wnt signaling acts through Lef1 to regulate neurogenesis in the zebrafish hypothalamus. We show that Lef1 is required for proneural and neuronal gene expression, and for neuronal differentiation in the posterior hypothalamus. Furthermore, we find that this process is dependent on Wnt8b, a ligand of the canonical pathway expressed in the posterior hypothalamus, and that both Wnt8b and Lef1 act to mediate β-catenin-dependent transcription in this region. Finally, we show that Lef1 associates in vivo with the promoter of *sox3,* which depends on Lef1 for its expression and can rescue neurogenesis in the absence of Lef1. The conserved presence of this pathway in other vertebrates suggests a common mechanism for regulating hypothalamic neurogenesis.

KEY WORDS: Zebrafish, Wnt, Lef1, Hypothalamus

INTRODUCTION

The hypothalamus is an evolutionarily conserved vertebrate brain structure responsible for regulation of the autonomic nervous system and endocrine hormone production. Although many specific neuronal populations in the adult hypothalamus have been well characterized, relatively little is known about the process through which these neurons are induced and specified during development. In zebrafish, where initial hypothalamus induction and patterning has been extensively studied, these events primarily occur in the first 18 hours of development (Varga et al., 1999; Woo and Fraser, 1995). The hypothalamus develops from the most ventral region of the anterior diencephalon, and is induced through identified molecular pathways such as Sonic Hedgehog and Nodal signaling. Specifically, Hedgehog signaling is required for inducing the anterior hypothalamus and Nodal signaling is required for the posterior hypothalamus (Chiang et al., 1996; Mathieu et al., 2002). After initial induction and patterning, the hypothalamus is regionalized into subdomains distinguished by specific gene expression patterns (Hauptmann and Gerster, 2000), but the upstream signals responsible for these subdivisions are unknown. In zebrafish, proneural markers begin to be expressed in specific regions of the hypothalamus by 18 hours post-fertilization (Mueller and Wullimann, 2002), but it is not clear which mature neuronal populations are labeled by these markers (Guo et al., 1999; Ross et al., 1992; Wilson et al., 1990). By contrast, the anatomical identities of specific neuronal populations in the hypothalamus of larval and adult zebrafish have been well characterized (Rink and Wullimann, 2001). Therefore, there is a gap in our understanding of the developmental signaling pathways between hypothalamic patterning and the eventual functional anatomy in the hypothalamus.

We are interested in the function of Wnt/β-catenin signaling in hypothalamic neurogenesis. Canonical Wnt signaling plays important roles in embryonic patterning, cell-fate determination, cell proliferation and cell differentiation during vertebrate development. Several previous studies have demonstrated roles for Wnt signals in specific aspects of central nervous system (CNS) formation (Logan and Nusse, 2004). In neural induction, Wnt signals from the paraxial mesoderm are required for the specification of posterior neural character (Nordstrom et al., 2002) during initial anteroposterior (AP) patterning. Later, this patterning is further refined into smaller subdivisions that also require Wnt signals from the posterior (Houart et al., 2002). Importantly, Wnt signaling induces posteriorisation during development of the zebrafish hypothalamus (Kapsimali et al., 2004). However, the required functions of canonical Wnt signals in later developmental steps are poorly understood, partly because of functional redundancy (Lekven et al., 2003).

Although the roles of some specific Wnt proteins in CNS development have been characterized (Brault et al., 2001; Buckles et al., 2004; Erter et al., 2001; Houart et al., 2002; Lee et al., 2000), they have primarily been defined in the context of general brain regions, such as the cerebellum or hippocampus. Wnt genes continue to be expressed in the brain at later embryonic stages, when they have been proposed to function in neuronal maturation, synapse formation, synaptic plasticity and axon guidance (Ciani and Salinas, 2005). However, the specific downstream targets of Wnt signaling during later embryogenesis remain unclear. In particular, there is little information on what functions Wnt signaling may have in the development of particular neuronal populations.

The nuclear response to canonical Wnt signals is mediated by the Lef/Tcf family of transcription factors, including lymphoid enhancer factor 1 (Lef1), which activate downstream genes by association with β-catenin (Eastman and Grosschedl, 1999). All Lef/Tcf proteins have highly similar DNA and β-catenin interaction domains, and there are no known differences in their affinities for these targets. In the absence of β-catenin, some members of the Lef/Tcf family can repress the transcription of target genes in cooperation with co-repressors such as Groucho and CtBP (Roose and Clevers, 1999). However, identified isoforms of Lef1 in zebrafish embryos lack a putative co-repressor interacting domain (Dorsky et al., 1999), and cannot substitute for the repressor function of Tcf3 in AP patterning (Dorsky et al., 2003), suggesting that Lef1 may function only as a transcriptional activator in the presence of β-catenin. Of the identified Lef/Tcf

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family members, only Lef1 has thus far been shown to play a required role in CNS neurogenesis (Galceran et al., 2000; van Genderen et al., 1994).

In zebrafish, Lef1 is expressed in multiple tissues during embryonic development, including the CNS (Dorsky et al., 1999). Removal of maternal and zygotic *lef1* function using a translation blocking morpholino oligonucleotide (MO) results in tail truncations and paraxial mesoderm defects (Dorsky et al., 2002). However, the expression and function of Lef1 at later stages in zebrafish remain uncharacterized. In the present study, we have investigated the role of Lef1 in the developing zebrafish brain using splice-blocking MOs and mutants. We show that *lef1* is expressed in the posterior hypothalamus after initial patterning but before the first neurons differentiate. In addition, we find that Wnt8b is expressed appropriately to function as a specific upstream modulator of Lef1 through the canonical pathway during hypothalamic development. We demonstrate through loss-of-function experiments that Wnt8b and Lef1 are required for the development of a specific neuronal population in the posterior hypothalamus, and signal through the canonical Wnt pathway in this region. These studies address for the first time the requirement for Wnt/β-catenin signaling in hypothalamic neurogenesis. In addition, analysis of downstream targets suggests a specific role for Lef1 in this later step of CNS development, in which it regulates a neurogenesis program by activating the expression of *sox3*, a gene required for neural competence.

MATERIALS AND METHODS

Fish strains and staging

Embryos were obtained from natural spawning of wild-type (AB*) or TOPdGFP (Dorsky et al., 2002) zebrafish lines. X8 deletion mutant embryos (line provided by Dr B. Riley) were identified by consistent phenotypes in 25% of embryos from crosses of heterozygous parents. All developmental stages in this study are reported in hours post-fertilization (hpf) at 28.5°C (Kimmel et al., 1995).

Morpholino injections

The *lef1* splice-blocking morpholino antisense oligonucleotide (MO) was obtained from Gene Tools (5′-ACTGCCTGGATGAAACACTT-ACATG-3′). The *wnt8b* translation-blocking MO (Riley et al., 2004) was kindly provided by Dr B. Riley. Both MOs were injected into one-cell stage wild-type or transgenic embryos at doses of 2 ng and 0.5 ng, respectively.

RT-PCR

Fifty wild-type embryos and *lef1* morphants were used for preparing RNA. Total RNA was isolated using Trizol reagent and standard protocols. Total RNA (1-5 μg/μl) was reverse transcribed by either random hexamers or a gene-specific primer using the Superscript first strand synthesis kit (Invitrogen) following the manufacturer's protocol. PCR was performed for 30-35 cycles using an annealing temperature of 55°C, and reactions were visualized on 1% agarose gels in TAE.

RNA injections

The *lef1* and *sox3* mRNAs were synthesized from *lef1*-pCS2+MT and *sox3*-pCS2+MT plasmids, respectively, using the SP6 mMessage mMachine transcription kit (Ambion). For mRNA rescue experiments, 100 pg of *lef1* mRNA and 20 pg of *sox3* mRNA were injected into one-cell stage wild-type embryos together with or without 2 ng of *lef1* MO.

In situ hybridization and immunohistochemistry

Probe synthesis and in situ hybridization were performed as described elsewhere (Oxtoby and Jowett, 1993). Single and double in situ hybridizations were carried out using digoxigenin- or fluorescein-labeled antisense RNA probes (Jowett, 2001) and visualized using BM Purple and Fast Red (Roche). The following RNA probes were used: *lef1* (Dorsky et al., 1999); *nk2.1a* (Rohr et al., 2001); *rx3* (Chuang et al., 1999); *emx2* (Morita et al., 1995); *sox3* (Kudoh et al., 2004); *zash1a* (Allende and Weinberg, 1994); *dlx2* (Akimenko et al., 1994); *isl1* (Okamoto et al., 2000); *wnt8b* (Kelly et al., 1995); *gfp* (Dorsky et al., 2002); *ngn1* (Blader et al., 1997); *olig2* (Park et al., 2002). Antibodies were obtained from the following sources: anti-pH3 (Upstate Biotechnology, 1:500), anti-GFP (Molecular Probes, 1:5000), anti-HuC/D (Molecular Probes, 1:500), anti-acetylated Tubulin (Sigma, 1:1000) and affinity-purified rabbit anti-Lef1 (Open Biosystems, 1:500). For immunostaining, embryos were fixed with 4% paraformaldehyde (PFA) for 3 hours at room temperature, and incubated with primary and secondary antibodies at 4°C overnight. For whole-mount photography after all staining methods, yolks and eyes of embryos were dissected. Hu, pH3 and AT-stained embryos were imaged on a confocal microscope, all other embryos and cryosections were imaged on a compound microscope.

TUNEL staining

For TUNEL analysis, 19 and 24 hpf embryos were fixed with 4% PFA for 4 hours at room temperature. Embryos were permeabilized with acetone at –20°C and washed twice with PBC (0.001% Triton X-100, 0.1% sodium citrate in PBS) for 10 minutes. Labeling for apoptotic cells was performed using In situ Cell Death Detection Kit (Roche) at 37°C for 1 hour, washed and mounted for fluorescent microscopic imaging.

ChIP

ChIP analysis was performed as described previously (Weinmann et al., 2001) with the following modifications. One-hundred embryos at 24-28 hpf were fixed in 1.85% formaldehyde for 15 minutes at room temperature, and then lysed in cell lysis buffer [10 mM Tris (pH 8.1), 10 mM NaCl, 0.5% NP-40, and protease inhibitors] by pipetting. For each immunoprecipitation, 5 μg of Lef1 antibody was conjugated to protein A beads.

The following primers were used for PCR after immunoprecipitation: *sox3*, 5′-AATTAGCCTTGCAGCCAATG-3′ and 5′-ATCGGAAGGG-GTTTCTCAAT-3′; *ngn1*, 5′-GGGCTCATTGGAGCAAGTTTGATT-3′ and 5′-CGCGGTAGCCTACATTACTGCACA-3′; *nacre*, 5′-GCAAT-TACCAAAGGCCCATCAGAC-3′ and 5′-ACTGGCTTACGGCTAA-CTAACGTT-3′.

Western blotting

Dechorionated embryos were homogenized in 4× sample buffer, subjected to 8% SDS-PAGE electrophoresis, and blotted onto PVDF membrane. Affinity-purified rabbit anti-Lef1 serum was applied at 1:2000 dilution, and anti-rabbit IgG-HRP (Molecular Probes) was applied at 1:10,000. The secondary antibody was visualized with an ECL reaction, using standard protocols. The same blot was stripped and re-probed with rabbit anti-β-catenin at 1:5000 dilution (Sigma), and the same secondary antibody.

RESULTS

The expression of *lef1* suggests a role in hypothalamic development

Although *lef1* is expressed both maternally and zygotically in early zebrafish embryos (Dorsky et al., 1999), the expression pattern at later embryonic stages has not been characterized. To assess later roles of *lef1* during brain development, we examined mRNA expression during late somitogenesis stages by in situ hybridization (Fig. 1). At 14 hpf, the only specific brain expression is in the midbrain and in the midbrain-hindbrain boundary (Fig. 1A). At 16 hpf, *lef1* expression begins in the ventral forebrain (Fig. 1B); by 19 hpf, it becomes restricted to the posterior hypothalamus (Fig. 1C). Expression in all these brain regions continues until 30 hpf (Fig. 1D-F). By examining cross-sections through the posterior hypothalamus, we observed that *lef1* is expressed in presumptive mitotic and post-mitotic cells located in the medial and lateral regions, respectively (Fig. 1G). Comparison of *lef1* expression with other known hypothalamic markers, such as *dlx2* (Fig. 2) and *hlx1* (not shown) led us to conclude that its expression was limited to transverse domain 4 and the ventral part of domain 5 (Fig. 1H), as defined by Hauptmann and Gerster (Hauptmann and Gerster, 2000).

The specific expression pattern of *lef1* therefore suggested that it may play an important role in the posterior hypothalamus following the initial steps of tissue induction and patterning.

If Lef1 acts in the canonical Wnt signaling pathway, we would expect a Wnt ligand to be expressed in close proximity to the *lef1*-expressing hypothalamic cells. The *wnt8b* gene, which encodes a ligand of the canonical pathway, is expressed in the forebrain during late somitogenesis stages (Kelly et al., 1995). We observed *wnt8b* expression in the posterior hypothalamus at 19 hpf in a region overlapping with and adjacent to *lef1* expression (Fig. 1I). Previous studies have shown that *lef1* is itself a target of Wnt signaling (Kengaku et al., 1998), and we also observed that that *lef1* was severely downregulated following injection with a previously published *wnt8b* MO (Riley et al., 2004). We therefore concluded that Wnt8b functions upstream of Lef1 expression during brain development.

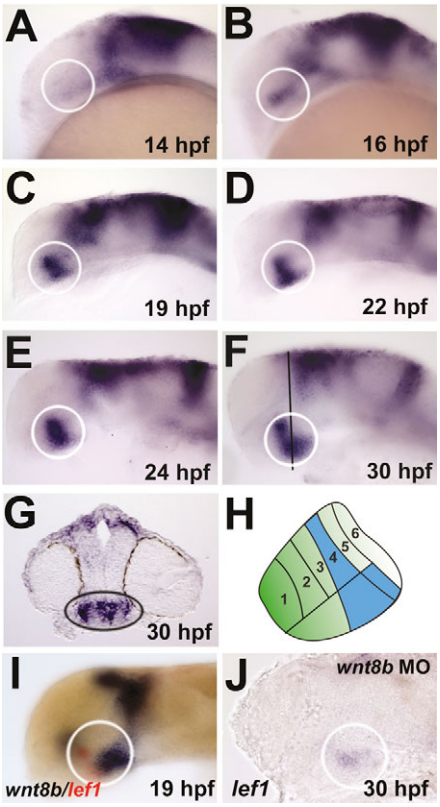

Fig. 1. *lef1* is expressed in the posterior hypothalamus during embryonic development. Lateral views are shown with anterior towards the left. White circles outline posterior hypothalamus. (**A**) At 14 hpf, *lef1* is strongly expressed in the dorsal midbrain, but does not show specific expression in the developing hypothalamus. (**B-F**) At 16 hpf, *lef1* expression first appears in the presumptive posterior hypothalamus, and this expression is maintained through 30 hpf. After 19 hpf, expression is present in dorsal and ventral regions of the posterior hypothalamus. Black line in F indicates plane of section in G. (**G**) Transverse section through the posterior hypothalamus (black oval) at 30 hpf, showing *lef1* expression in both the medial mitotic cells and the lateral postmitotic cells of the posterior region. (**H**) Schematic depiction of *lef1* expression domain in 30 hpf zebrafish hypothalamus. Numbered regions are based on those of Hauptmann and Gerster (Hauptmann and Gerster, 2000), and *lef1* expression is shown in blue. (**I**) At 19 hpf, *wnt8b* (blue) and *lef1* (red) show overlapping and adjacent expression in the posterior hypothalamus. (**J**) In *wnt8b* morphants, *lef1* expression is reduced throughout the brain.

We next asked whether the expression of specific proneural and neuronal genes overlapped with *lef1* in the posterior hypothalamus. We examined the expression of *sox3*, which encodes an HMG-box transcription factor in the SoxB1 family, members of which function at an early step in the process of neurogenesis (Kan et al., 2004). At 19 hpf, *sox3* expression did not overlap with *lef1* in the posterior hypothalamus (Fig. 2A,B). By 22 hpf, we observed co-expression of the two genes, which was maintained through 30 hpf (Fig. 2E,F). The *zash1a* gene (*ascl1a* – Zebrafish Information Network), which encodes a proneural bHLH transcription factor, was previously shown to be expressed in the posterior hypothalamus (Allende and Weinberg, 1994). We found that *zash1a* was not co-expressed with *lef1* in the posterior hypothalamus at 24 hpf (Fig. 2C,D), but co-expression was observed beginning at 26 hpf and continuing through 30 hpf (Fig. 2G,H). For both *sox3* and *zash1a*, we observed co-expression with *lef1* in medial progenitors and more lateral differentiated neurons.

By contrast, two other genes are co-expressed with *lef1* only in differentiated hypothalamic neurons at 30 hpf (Fig. 2I-L). The *dlx2* gene, which is involved in forebrain regional specification, is also expressed in transverse domain 4 of the posterior hypothalamus (Hauptmann and Gerster, 2000). The expression of *dlx2* primarily in postmitotic neurons suggests that it might act to regulate neuronal differentiation in this region, rather than playing an earlier role in progenitor specification. Finally, *isl1* labels specific populations of differentiated neurons throughout the embryo, and was detected in the posterior hypothalamus at 30 hpf.

Lef1 is not required for induction or AP patterning of the hypothalamus

To determine the required function for *lef1* during hypothalamic development, we used two methods to inactivate zygotic gene function. First, a splice-blocking MO was designed against an intron-exon boundary in the region encoding the DNA-binding HMG box. This region was targeted because exon-skipping, a potential outcome of splice-blocking MOs, would create a protein unable to bind DNA. In fact, RT-PCR analysis of injected embryos showed a smaller product, indicating the presence of a cryptic splice donor in the preceding exon (see Fig. S1 in the supplementary material). Sequencing of this product confirmed a small deletion, which resulted in a shifted open reading frame. Furthermore, RT-PCR (see Fig. S1 in the supplementary material) and in situ hybridization (not shown) indicated that *lef1* mRNA levels rapidly decreased following MO injection, an outcome that could be due to either nonsense-mediated decay or lack of auto-activation of *lef1* transcription (Kengaku et al., 1998). Second, we examined embryos homozygous for the X8 deletion mutation, generated by Dr B. Riley. PCR and linkage analysis shows that X8 is a deletion in chromosome 1 with one end just distal to *msxB* and the other end proximal to *lef1*, a distance of 2-8 cM (Phillips et al., 2006). Although X8 probably removes many genes in addition to *lef1*, the only other identified gene in this region is *msxB*, which is not expressed in the developing hypothalamus. Importantly, in all following experiments both MOs and the X8 mutation produced identical hypothalamic phenotypes.

Ventral midline CNS cells in the forebrain differentiate into hypothalamus anteriorly and floor plate posteriorly as a result of Nodal and Wnt signaling (Kapsimali et al., 2004). After the initial AP subdivision of ventral midline CNS fates, these signals also affect subsequent AP patterning within the hypothalamus (Kapsimali et al., 2004; Mathieu et al., 2002). To examine whether Lef1 is also required for AP patterning of the hypothalamus, we

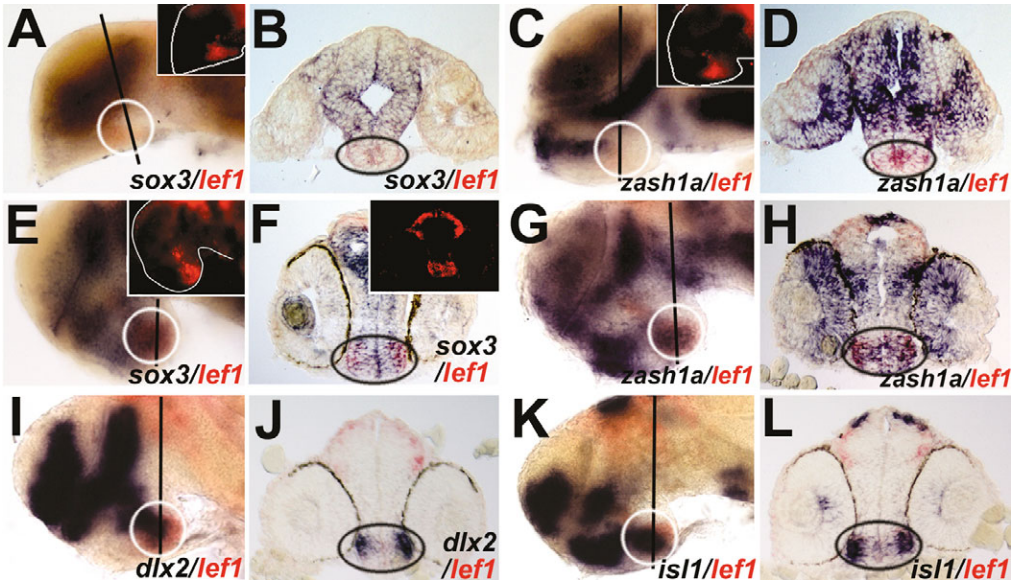

Fig. 2. *lef1* is co-expressed with proneural and neuronal markers in the posterior hypothalamus. White circles outline posterior hypothalamus and inset panels show *lef1* mRNA expression from Fast Red fluorescence. Lines in whole-mount images show plane of corresponding cross-sections on the right. In cross-sections, black ovals outline posterior hypothalamus. (**A**,**B**) At 19 hpf, *sox3* (blue) is not co-expressed with *lef1* (red) in the posterior hypothalamus. (**C**,**D**) At 24 hpf, *zash1a* (blue) is not co-expressed with *lef1* (red) in the posterior hypothalamus. (**E-H**) By 30 hpf, *sox3* and *zash1a* are co-expressed with *lef1* in both medial progenitors and lateral postmitotic neurons of the posterior hypothalamus. (**I-L**) By contrast, *dlx2* and *isl1* are co-expressed only with *lef1* in lateral postmitotic neurons.

performed in situ hybridization for specific patterning markers. We investigated hypothalamic AP patterning in wild-type embryos, *lef1* morphants and X8 mutants by comparing the expression of *nk2.1a* (titf1a – Zebrafish Information Network), *rx3* and *emx2*. The *nk2.1a* gene is a marker for the entire hypothalamus (Rohr et al., 2001), whereas *rx3* and *emx2*, respectively, mark the anterior and posterior hypothalamus (Chuang et al., 1999; Mathieu et al., 2002). As opposed to the severe defects observed in zebrafish *axin1* mutants (Kapsimali et al., 2004), all three markers were still expressed appropriately at 30 hpf in *lef1* morphants (Fig. 3) and X8 mutants (see Fig. S2 in the supplementary material), suggesting that the regional identity of posterior hypothalamus was unchanged.

Lef1 is required for proneural and neuronal gene expression in the posterior hypothalamus

The above observations, coupled with the expression pattern of *lef1* in the hypothalamus, suggested that this gene may play a role in a later step of hypothalamic development. Such a role would be consistent with previous studies demonstrating that Wnt signals can regulate neurogenesis in the vertebrate midbrain and hindbrain (Amoyel et al., 2005; Castelo-Branco et al., 2003). To determine whether Lef1 function is required for expression of the marker genes listed previously, we analyzed loss-of-function embryos. We found that expression of *sox3* and *zash1a* was absent in the posterior hypothalamus at 24 and 28 hpf, respectively, in both *lef1* morphants and X8 mutants (Fig. 4A-H; see Fig. S2 in the supplementary material). In addition, *dlx2* and *isl1* were also not expressed in the posterior hypothalamus of *lef1* morphants and X8 mutants at 30 hpf (Fig. 4I-P; see Fig. S2 in the supplementary material). All of these markers were expressed relatively normally in other forebrain regions. In addition, *ngn1* (*neurog1* – Zebrafish Information Network) and *olig2*, which are expressed posterior and dorsal to *lef1* in the posterior tuberculum, were not altered in *lef1* morphants (Fig. 4Q-T). Thus, expression of proneural and neuronal genes in the *lef1*-positive domain of posterior hypothalamus specifically requires *lef1* function. These data suggest that Lef1 may act to regulate the development of a specific population of hypothalamic neurons.

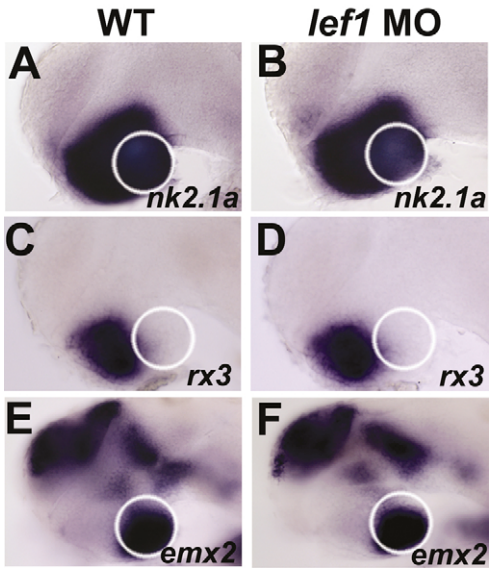

Fig. 3. Lef1 is not required for molecular markers of hypothalamus identity or AP patterning. White circles outline posterior hypothalamus. (A,C,E) Uninjected embryos. (B,D,F) Embryos injected with 2 ng of *lef1* MO. (**A**,**B**) *nk2.1a* expression in the entire hypothalamus is unaffected in *lef1* morphants at 30 hpf. (**C**,**D**) *rx3* is still expressed in the anterior hypothalamus in *lef1* morphants at 30 hpf. (**E**,**F**) *emx2* is still expressed in the posterior hypothalamus of *lef1* morphants at 30 hpf.

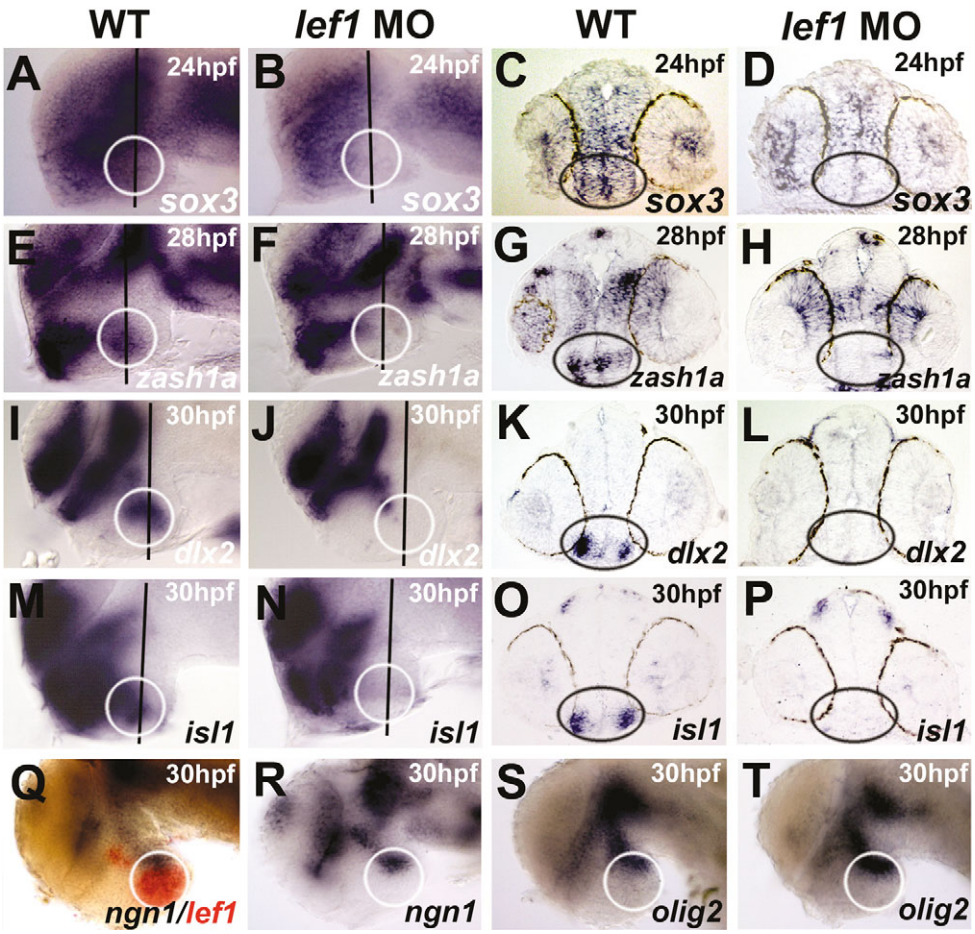

Fig. 4. Lef1 is required for proneural and neuronal gene expression in the posterior hypothalamus. White circles outline posterior hypothalamus in whole-mount views, and black ovals outline posterior hypothalamus in cross-sections. Lines in whole-mount images show plane of corresponding cross-sections. (**A-D**) Expression of *sox3* is absent in the posterior hypothalamus of *lef1* morphants at 24 hpf. (**E-H**) Expression of *zash1a* is absent in the posterior hypothalamus of *lef1* morphants at 28 hpf. (**I-P**) Expression of *dlx2* and *isl1* are absent in the posterior hypothalamus of *lef1* morphants at 30 hpf. (**Q-T**) Expression of *ngn1* and *olig2*, which are expressed in the posterior tuberculum, is unaffected in the posterior tuberculum of *lef1* morphants.

Rescue of marker gene expression by mRNA injection

To confirm that the phenotypes in *lef1* morphants were specific to the *lef1* gene and not due to other side-effects, we attempted to rescue hypothalamic gene expression by co-injection of *lef1* mRNA lacking the MO target sequence. We first titrated the dose of *lef1* mRNA to find a concentration that does not produce phenotypes when overexpressed, and observed normal development in embryos injected with 100 pg of mRNA. Co-injection of 100 pg of *lef1* mRNA rescued the expression of *zash1a*, *dlx2* and *isl1* at 30 hpf in the posterior hypothalamus of *lef1* morphants (Table 1; see Fig. S3 in the supplementary material). This result, combined with the similar phenotypes produced by MO injection and the X8 deletion, led us to conclude that the absence of gene expression in *lef1* morphants is specific to *lef1* loss of function.

Of all the markers we analyzed, *sox3* is expressed at the earliest time in the posterior hypothalamus and functions at the earliest step of neurogenesis (Kan et al., 2004). Furthermore, another *soxB1* family gene, *sox2*, has been shown to be downstream of canonical Wnt signaling in the *Xenopus* retina (Van Raay et al., 2005). We therefore investigated whether *sox3* mRNA could rescue the expression of the other proneural and neuronal markers in *lef1* morphants. Co-injection of 20 pg of *sox3* mRNA with the *lef1* MO rescued *zash1a*, *dlx2* and *isl1* expression at 30 hpf in the posterior hypothalamus (Table 1; see Fig. S3 in the supplementary material). These results led us to conclude that Lef1 may act through Sox3 to establish a program of neurogenesis in the posterior hypothalamus, resulting in eventual differentiation of a discrete neuronal population.

Lef1 is required for neurogenesis in the posterior hypothalamus

We also examined later effects on neuronal differentiation in *lef1* morphants. Hu proteins, which mark all postmitotic neurons, are expressed in the posterior hypothalamus at 36 hpf. We observed a specific and complete loss of Hu expression in the posterior

Table 1. Rescue of *lef1* MO phenotypes with *sox3* or *lef1* mRNA

Treatment	*zash1a* (%)	*dlx2* (%)	*isl1* (%)
None	100 (*n*=40)	100 (*n*=40)	100 (*n*=40)
lef1 MO	9 (*n*=109)	9 (*n*=135)	9 (*n*=135)
lef1 MO + *lef1* mRNA	61 (*n*=100)	60 (*n*=100)	61 (*n*=100)
lef1 MO + *sox3* mRNA	70 (*n*=104)	69 (*n*=104)	68 (*n*=104)

lef1 MO (2 ng), 20 pg of *sox3* mRNA and 100 pg of *lef1* mRNA were injected into one-cell stage embryos. Results of phenotypic rescue by the mRNA were obtained performing in situ hybridization for *zash1a*, *dlx2* and *isl1*. *P*-values were measured by Student's *t*-test comparing the results of single *lef1* MO injection to co-injection with either *sox3* mRNA or *lef1* mRNA for each gene in situ hybridization ($P<0.001$ in all comparisons).

hypothalamus of morphants (Fig. 5A-D). Interestingly, a subset of Hu-positive neurons in the posterior hypothalamus continue to express Lef1 protein even at 36 hpf (Fig. 5E), suggesting that Lef1 may play an additional later role in their differentiation or function. By 48 hpf, specific axonal populations are visible in the posterior hypothalamus by acetylated tubulin staining. We observed a loss of these axons in *lef1* morphants (Fig. 5F,G), and although the staining method used did not allow us to identify these axons as afferents or efferents, this result was consistent with decreased neuronal differentiation in this region.

Lef1 is not necessary for cell proliferation or survival in the posterior hypothalamus

The changes we observed in gene expression and neuronal differentiation led us to investigate whether hypothalamic progenitors exhibited changes in proliferation or apoptosis when Lef1 function was lost. First, we found expression of *pcna* mRNA (not shown) and phospho-histone H3 (pH3) in the posterior hypothalamus of morphants (Fig. 6A-L), indicating that proliferating cells were still present. The percentage of pH3-positive cells at 19 hpf (Fig. 6C,D) in the posterior hypothalamus was 7.5±2.0% (s.d., n=15) in controls compared with 5.4±1.1% (n=15) in morphants. At 24 hpf (Fig. 6G,H), there were 13±1.3% (n=15) positive cells in controls, compared with 11±2.1% (n=15) cells in morphants. At 30 hpf (Fig. 6K,L), we found 7.1±1.0% (n=15) pH3-positive cells in controls and 6.7±0.8% (n=15) in morphants. In addition, total cell number in the posterior hypothalamus was not significantly affected at any stage. Analysis of apoptosis by TUNEL staining showed no additional labeling in the hypothalamus of morphants at 19 or 24 hpf (Fig. 6M-P). These results, combined with the lack of Hu staining observed in morphants, suggest that the small decrease in pH3 index reflects a slower cell cycle time rather than premature differentiation or cell death. Therefore, although Lef1 may be required for the proper rate of cell division, it is not required for proliferation in general and the reduced proliferation rate alone cannot explain the complete lack of proneural and neuronal markers we observed in morphants. Our results are consistent with a model where in the absence of Lef1 function, dividing hypothalamic progenitors remain in an immature undifferentiated state and fail to acquire neural competence.

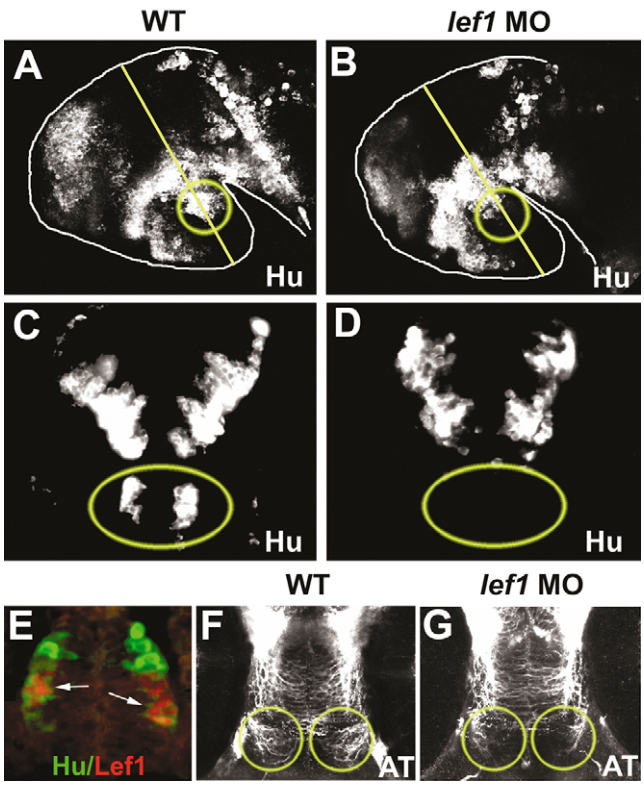

Fig. 5. Lef1 is required for neuronal differentiation in the posterior hypothalamus. (**A**,**B**) Confocal projections through the hypothalamus of whole-mount 36 hpf brains stained for HuC/D, a pan-neuronal marker. Owing to differences in mounting, neurons outside the hypothalamus may not be visible. (**C**,**D**) Cross-sections of 36 hpf brains through the plane marked by the lines in A,B. A specific population of Hu-positive neurons (yellow circles and ovals) is absent in the posterior hypothalamus of *lef1* morphants (B,D). (**E**) Hu (green) is co-expressed with Lef1 protein (red) in a subset of posterior neurons at 36 hpf. (**F**,**G**) Acetylated tubulin (AT) staining labels specific axons in the posterior hypothalamus (yellow circles) that are reduced in *lef1* morphants at 48 hpf. Confocal projections through the ventral side of the hypothalamus are shown, with anterior towards the top.

Wnt8b regulates gene expression in the posterior hypothalamus similarly to Lef1

In addition to our finding that *wnt8b* was expressed near *lef1* during early hypothalamic development (Fig. 1I), we observed continued *wnt8b* expression in the posterior hypothalamus at 30 hpf in a region overlapping with and adjacent to *lef1* expression (Fig. 7A,B). To test whether *wnt8b* and *lef1* function are similarly required for hypothalamic neurogenesis, we examined *wnt8b* morphants for proneural and neuronal markers. In support of our hypothesis that Wnt8b functions upstream of Lef1 in hypothalamic development, we found that injection of *wnt8b* MO led to absence of *sox3*, *zash1a*, *dlx2* and *isl1* expression in the posterior hypothalamus at 30 hpf (Fig. 7C-F).

Wnt8b and Lef1 regulate β-catenin-mediated transcription in the posterior hypothalamus

To test whether both Wnt8b and Lef1 act in the canonical Wnt pathway, we used a transgenic reporter for β-catenin-mediated transcription (TOPdGFP), in which GFP is activated in a Lef1-dependent manner (Dorsky et al., 2002). First, we examined whether *gfp* mRNA expression overlapped with *wnt8b* and *lef1* expression in the posterior hypothalamus of TOPdGFP embryos at 24 hpf, shortly after *sox3* expression first appears in this region. Double in situ hybridization on 24 hpf TOPdGFP embryos showed adjacent expression of *gfp* and *wnt8b* in the posterior hypothalamus. (Fig. 7G,H). In addition, *gfp* was expressed in a overlapping pattern with *lef1* in the posterior hypothalamus (Fig. 7I). The relative expression patterns of these three genes were maintained until 30 hpf (not shown).

We next asked whether TOPdGFP expression is disrupted in the posterior hypothalamus when either *wnt8b* or *lef1* function is removed. To address this issue, we performed immunohistochemistry for GFP protein after injecting *wnt8b* or *lef1* MOs into TOPdGFP embryos. Removal of both *wnt8b* and *lef1* resulted in the loss of GFP in the posterior hypothalamus at 24 and 30 hpf (Fig. 7J-O). These results were consistent with our observations that *lef1* expression coincides with *gfp* mRNA in this region, and suggested that although Wnt8b may function as a secreted ligand, Lef1 probably functions cell-autonomously. Because *lef1* expression was downregulated in *wnt8b* morphants, we cannot determine whether Wnt8b normally acts only to induce *lef1*,

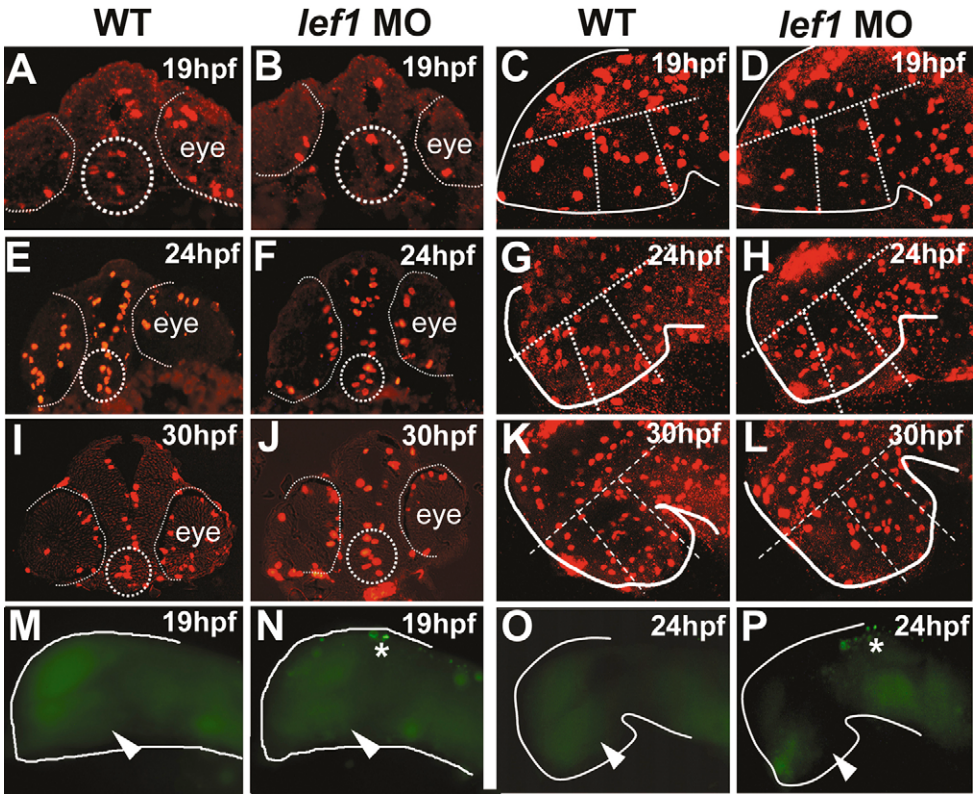

Fig. 6. Analysis of proliferation and apoptosis in *lef1* morphants. (**A-L**) In *lef1* morphants, pH3-positive cells are observed in the posterior hypothalamus at 19, 24 and 30 hpf, indicating that proliferating cells are still present in this region. (A,B,E,F,I,J) Cross-sections through posterior hypothalamus (outlined by dotted circles). (C,D,G,H,K,L) Confocal projections of pH3-stained embryos used for quantitative analysis. The central region outlined by broken lines was defined as posterior hypothalamus, based on morphology in bright-field views. The region farthest to the right was excluded from counts because it does not express *lef1* and is unaffected in *lef1* morphants (Fig. 4Q-T). (**M-P**) Apoptotic cells identified by TUNEL staining are absent in the posterior hypothalamus at 19 hpf and 24 hpf in wild-type and *lef1* MO-injected embryos (arrowheads). Asterisks indicate apoptotic cells in the dorsal brain of *lef1* morphants.

or also to signal through Lef1 in target gene activation. However, our data suggest that both genes are required in the same pathway mediating hypothalamic neurogenesis.

Chromatin immunoprecipitation identifies *sox3* as a binding target of Lef1 in vivo

Our analysis of proneural and neuronal markers indicating that progenitor genes may be downstream targets of *lef1* gene function, and our observation that *sox3* mRNA could rescue the *lef1* MO phenotype (Table 1; see Fig. S3 in the supplementary material), led to the hypothesis that *sox3* transcription could be directly regulated by β-catenin and Lef1. We therefore employed chromatin immunoprecipitation (ChIP) assays to determine whether the *sox3* upstream regulatory region binds to Lef1 protein in vivo. For immunoprecipitation, we used a polyclonal antibody from our laboratory, raised against zebrafish Lef1 (also used in Fig. 5E). This antibody recognizes a single band of ~50-55 kDa in lysates from wild-type 24 hpf embryos, and this band is absent in X8 deletion mutant embryo lysates, indicating that it specifically detects Lef1 protein (Fig. 8A).

To identify putative Lef1-binding sites in upstream regulatory sequences of *sox3*, we analyzed genomic sequence data from the Sanger Centre zebrafish genome assembly and found several consensus sites within 10 kb upstream of the *sox3* transcription start site. PCR primers designed to flank these putative sites were able to amplify products from chromatin extracts of 24 hpf embryos. Following sonication, immunoprecipitation and de-crosslinking, we were able to amplify one of these fragments from Lef1 antibody precipitated extracts, but not from controls with no antibody, or more importantly from X8 mutant extracts that lack Lef1 protein (Fig. 8B). The fragment that specifically interacted with Lef1 protein was located ~6.5 kb upstream of the *sox3* transcription start, and contains a consensus binding site in a region that is conserved between zebrafish and *Fugu*. As a positive control, we were also able to immunoprecipitate binding sites in the promoters of *nacre* (*mitfa* – Zebrafish Information Network) and *ngn1* (Fig. 8B), both previously identified as β-catenin target genes (Dorsky et al., 2000; Hirabayashi et al., 2004). We therefore conclude that Lef1 specifically interacts with an upstream regulatory region of *sox3* in 24 hpf zebrafish embryos, and thus may be a direct transcriptional activator of this gene in the posterior hypothalamus.

DISCUSSION

In this study, we have revealed a specific role for canonical Wnt signaling during hypothalamic neurogenesis by analyzing the function of zebrafish Lef1, an essential mediator of the pathway. In Lef1 loss-of-function embryos, proneural and neuronal markers and differentiated neurons are absent from the posterior hypothalamus, indicating a requirement for Lef1 in the development of these cells. We have also shown that Wnt8b and Lef1 both act through the canonical Wnt pathway in this region. Our data indicate that the activation of *sox3* downstream of canonical Wnt signaling drives a neurogenesis program during zebrafish posterior hypothalamus development, eventually leading to the differentiation of a specific neuronal population (Fig. 9).

Specific expression of canonical Wnt pathway components in the posterior hypothalamus

The expression of zebrafish *lef1* begins at 16 hpf in the ventral forebrain, and continues until 30 hpf in the posterior hypothalamus. Of all known zebrafish *lef/tcf* family genes, *lef1* is the only member expressed in this particular brain region, suggesting that it may have a unique function in hypothalamic development. We have found that *lef1* is expressed in both the progenitors and neurons of the posterior hypothalamus, suggesting that it may function to regulate a program of neurogenesis. Examination of a canonical Wnt pathway reporter,

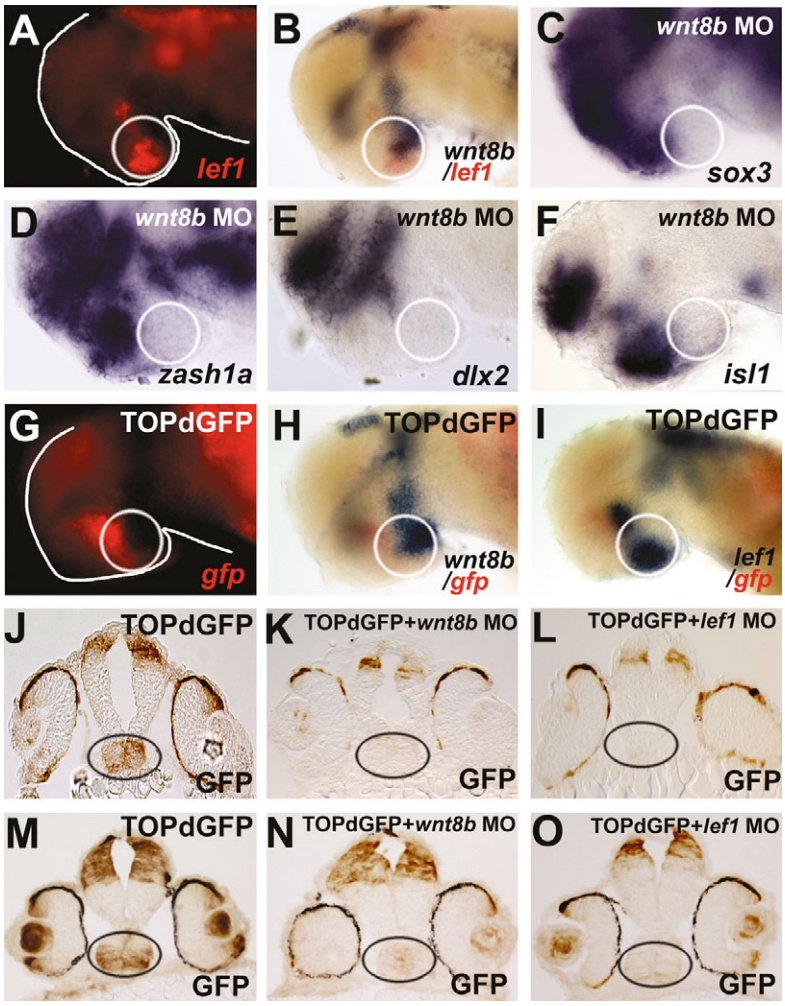

Fig. 7. Wnt8b and Lef1 act through the canonical Wnt pathway in the posterior hypothalamus. White circles outline posterior hypothalamus in whole-mount views, and black ovals outline posterior hypothalamus in cross-sections. (**A**,**B**) At 30 hpf, *wnt8b* (blue) and *lef1* (red) show overlapping and adjacent expression in the posterior hypothalamus. (**C-F**) In *wnt8b* morphants, *sox3*, *zash1a*, *dlx2* and *isl1* expression are specifically reduced in the posterior hypothalamus at 30 hpf. (**G-I**) A transgenic reporter for β-catenin-mediated transcription (TOPdGFP) shows mRNA expression in the posterior hypothalamus (red) at 24 hpf. In the posterior hypothalamus, *wnt8b* expression (blue-H) partially overlaps with *gfp*, whereas *lef1* (blue-I) almost completely overlaps with *gfp*. (**J-O**) In TOPdGFP embryos, GFP protein (brown) is expressed in the posterior hypothalamus. At 24 hpf (J-L) and 30 hpf (M-O), this expression is absent in *wnt8b* and *lef1* morphants.

TOPdGFP, indicates that this pathway is active where *lef1* is expressed. Furthermore, we have identified a gene encoding an upstream ligand of the canonical Wnt pathway, *wnt8b*, expressed adjacent to *lef1* in the posterior hypothalamus and required for *lef1* expression. Together, these findings indicate that β-catenin and Lef1 may interact in hypothalamic progenitors to activate target genes involved in neurogenesis.

Lef1 is required for neurogenesis in the posterior hypothalamus

Canonical Wnt signaling is required for the initial induction and subsequent AP pattering of the hypothalamus (Wilson and Houart, 2004). Our data indicate that Lef1 does not regulate either of these events, based on the unaffected expression of markers such as *nk2.1a*, *rx3*, *emx2*, *ngn1* and *olig2*, and the presence of anterior hypothalamic neurons in morphants and mutants. By contrast, we find a specific loss of genes expressed in progenitors (*sox3* and *zash1a*) and in postmitotic neurons (*dlx2* and *isl1*) in the posterior hypothalamus of these embryos. In addition, we observed continued cell proliferation and no increase in apoptosis, suggesting that progenitor cells remain in an undifferentiated state and fail to be specified as neural precursors. Because we observed a complete loss of Hu-positive cells in the posterior hypothalamus in *lef1* morphants, we conclude that neurons are not mis-specified to alternate fates by 36 hpf, and that Lef1 function is required for the entire process of neurogenesis in this region. We cannot determine whether neurogenesis has been inhibited or merely delayed in the absence of Lef1; however, analysis of neuronal markers at 36 hpf (not shown) indicates it is delayed by at least 6 hours. In either case, our data suggest that Lef1 regulates the proper timing of neurogenesis in the hypothalamus. Although it has been shown previously that Lef1 is required for the generation of neuronal populations in the mouse cortex and midbrain (Galceran et al., 2000; van Genderen et al., 1994), our data are the first to demonstrate that hypothalamic neurogenesis is regulated by Lef1.

Lef1 functions downstream of Wnt8b to regulate neurogenesis through Sox3

Multiple Wnt proteins are involved in canonical signaling, but it has been difficult to study the specific function of individual Wnts because of their redundant functions in CNS development. In this case, we have found that Wnt8b is uniquely positioned to act upstream of Lef1 in the posterior hypothalamus. Our studies provide two sets of data indicating that canonical Wnt signaling through Wnt8b is required for neurogenesis. First, when we eliminated *wnt8b* function proneural and neuronal marker gene expression was lost, producing a similar phenotype to *lef1* morphants. Second, expression of TOPdGFP was specifically eliminated in the posterior hypothalamus in both *lef1* and *wnt8b* morphants. We were unable to rescue neurogenesis in *wnt8b* morphants by *sox3* overexpression, perhaps owing to additional roles for *wnt8b* in embryonic development. Indeed, Wnt8b may signal through other Tcf proteins,

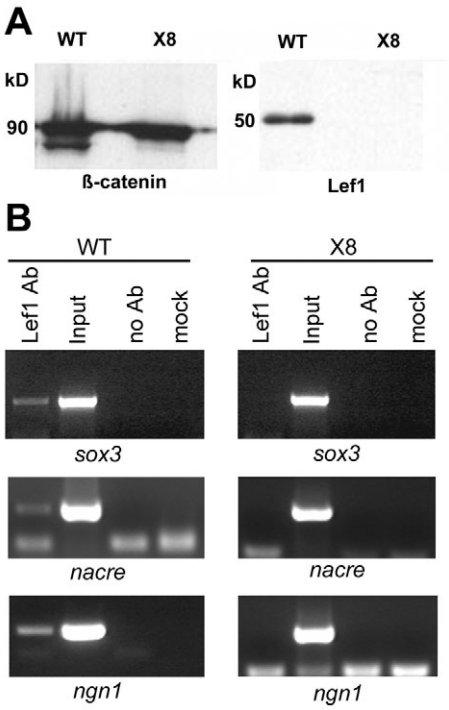

Fig. 8. Lef1 associates with the *sox3* promoter in vivo. (**A**) Western blot of whole-embryo lysates probed with anti-β-catenin and Lef1 polyclonal antibodies. The Lef1 band is specifically absent in X8 mutants, whereas the positive control β-catenin band is present. (**B**) ChIP analysis of whole-embryo lysates shows that the Lef1 antibody can immunoprecipitate DNA fragments containing Lef-binding sites in the upstream regulatory region of *sox3*, *nacre* and *ngn1*. These fragments do not precipitate in controls lacking antibody or chromatin (mock). In lysates from X8 mutants that lack Lef1 protein, the antibody is unable to precipitate these fragments, suggesting that the interaction is specific to Lef1.

including Tcf7 which is also expressed in the forebrain (Veien et al., 2005). Our experiments therefore cannot determine whether after Wnt8b initiates *lef1* expression, a different Wnt signal then activates the pathway through Lef1. However the continued expression of Wnt8b in the posterior hypothalamus through 30 hpf suggests that it might be required for both functions.

Our data indicate that Lef1 protein binds directly to upstream regulatory elements of the *sox3* gene in 24 hpf zebrafish embryos. Combined with the requirement for *lef1* in the hypothalamic expression of *sox3*, and the ability of *sox3* to rescue the loss of Lef1 function, we propose that β-catenin and Lef1 cooperate to directly activate *sox3* transcription in the posterior hypothalamus. A more detailed promoter analysis, including mutagenesis of potential binding sites, will be necessary to prove conclusively that *sox3* directly requires Lef1 for its activation in posterior hypothalamic progenitors. However, we believe that this model most accurately explains the phenotypes we observe in our embryos. Evidence from other species indicates that SoxB1 factors are required for the acquisition of neural potential (Graham et al., 2003; Kan et al., 2004). Therefore, activation of *sox3* by Lef1 could initiate a program of neurogenesis, and subsequently allow for cell cycle exit and neuronal differentiation. Our model would also be consistent with results obtained in the *Xenopus* retina, where elimination of Fz5 function results in loss of both *sox2* and proneural gene expression (Van Raay et al., 2005).

Evolutionary conservation of Sox3 and Wnt function in posterior hypothalamic development

In contrast to the larva and adult, hypothalamic neuronal identity and anatomy has been poorly characterized during zebrafish embryogenesis. In the present study, we have shown that a specific population of neurons in the posterior hypothalamus differentiates between 22 and 36 hpf, and that its development depends on Lef1 function. However, we do not know the ultimate fate of the neurons that we have identified in this study. Several neurotransmitters have been identified in the hypothalamus in zebrafish larvae and adults (Doldan et al., 1999; Poon et al., 2003; Ross et al., 1992; Teraoka et al., 2004). In addition, dopaminergic neurons project from the tuberal hypothalamus to the subpallium in adults, but it remains unclear when the projection arises (Rink and Wullimann, 2001). Previous studies have reported that neurotransmitter expression and axonogenesis in the posterior hypothalamus appears between 36 and 48 hpf, although proneural genes are expressed about one day earlier (Clemente et al., 2004; Hauptmann and Gerster, 2000; Wilson et al., 1990; Wullimann and Mueller, 2004). Intriguingly, a recent report shows specific expression of the secreted hormones AGRP and PMOC as early as 24 hpf in the posterior hypothalamus (Song et al., 2003). This result, combined with the similar expression of *dlx2* in the mouse hypothalamus (Bulfone et al., 1993) and in zebrafish Lef1-dependent neurons, suggests that these cells may contribute to the infundibulum or neurohypophysis.

Significantly, at least three of the genes analyzed in this study in addition to *dlx2* show similar expression domains in the mammalian posterior hypothalamus. The expression of *wnt8b* has previously been reported in the mammillary and retromammillary hypothalamus of mice and humans (Lako et al., 1998). Both the endogenous *lef1* gene and a reporter knocked into the mouse *lef1* locus shows expression in the posterior hypothalamus, although the precise location has not been characterized (Galceran et al., 2000).

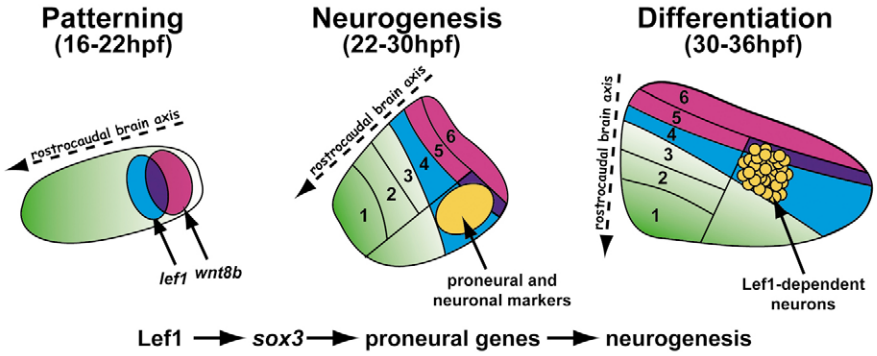

Fig. 9. Summary and model. During A/P patterning of the hypothalamus (16-22 hpf), lef1 and wnt8b are expressed in posterior regions. Later, when neurogenesis begins in the posterior hypothalamus (22-30 hpf), *lef1* is required for the expression of *sox3*, which in turn regulates proneural and neuronal gene expression. After 30 hpf, a specific population of Lef1-dependent neurons differentiates in this region. Morphology of the hypothalamus is adapted from Hauptmann and Gerster (Hauptmann and Gerster, 2000).

Finally, not only is *sox3* expressed in the mammalian infundibulum (Solomon et al., 2004), specific mutations in this gene lead to defects in infundibular hypoplasia and associated hypopituitarism in both mice and humans (Rizzoti et al., 2004; Woods et al., 2005). Our data suggest that a pathway containing Wnt8b, Lef1 and Sox3 may be an important conserved mechanism for driving a program of neurogenesis in the posterior hypothalamus and promoting normal endocrine hormone function.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/22/4451/DC1

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