**ABSTRACT**

In the developing brain, growth and differentiation are intimately linked. Here, we show that in the zebrafish embryo, the homeodomain transcription factor Rx3 coordinates these processes to build the tuberal/anterior hypothalamus. Analysis of rx3 chk mutant/rx3 morphant fish and EdU pulse-chase studies reveal that rx3 is required to select tuberal/anterior hypothalamic progenitors and to orchestrate their anisotropic growth. In the absence of Rx3 function, progenitors accumulate in the third ventricular wall, die or are inappropriately specified, the shh+ anterior recess does not form, and its resident pomc+, fflb+ and otpb+ Th1+ cells fail to differentiate. Manipulation of Shh signalling shows that Shh coordinates progenitor cell selection and behaviour by acting as an on-off switch for rx3. Together, our studies show that Shh and Rx3 govern formation of a distinct progenitor domain that elaborates patterning through its anisotropic growth and differentiation.

**KEY WORDS:** Hypothalamus development, Anterior hypothalamus, Rx3, Sonic hedgehog, Tuberal hypothalamus, Zebrafish hypothalamus

**INTRODUCTION**

The hypothalamus is an ancient part of the ventral forebrain. It centrally regulates homeostatic processes that are essential to survival and species propagation, including autonomic regulation of energy balance, growth, stress and reproduction. Such adaptive functions have also been largely conserved (reviewed by Bedont et al., 2015; Biran et al., 2015; Burbridge et al., 2016; Pearson and Placzek, 2013; Puelles et al., 2012).

The mechanisms through which secreted signalling ligands and transcription factors define and build hypothalamic territories and cells remain enigmatic (see Bedont et al., 2015; Puelles et al., 2012; Pearson and Placzek, 2013). Models based on the uniform growth and differentiation of patterned territories do not account for the complex spatial patterns of the hypothalamus or the protracted period of hypothalamic neuronal differentiation and, at present, little is known about how early patterning events are elaborated over time. In the hypothalamus, distinct neural progenitor domains that form around the third (diencephalic) ventricle (3V) are not as well-characterized as those in other regions of the CNS. Moreover, the third ventricle is sculpted into the infundibular, optic, and other smaller and ill-defined recesses in mammals (Amat et al., 1992; O’Rahilly and Muller, 1990), and lateral (LR), posterior (PR) and anterior (AR) recesses in zebrafish (Wang et al., 2009, 2012). Three unexplored questions are when such hypothalamic recesses form, whether they are composed of distinct progenitor cells and whether their appearance correlates with the emergence of particular neuronal subsets.

The paired-like homeodomain transcription factor Rax (also known as Rx) and its fish orthologue, rx3, are expressed within retinal and hypothalamic progenitors (Bailey et al., 2004; Bielen and Houart, 2012; Cavodeassi et al., 2013; Chuang et al., 1999; Furukawa et al., 1997; Lu et al., 2013; Mathers et al., 1997; Stigloher et al., 2006; Medina-Martinez et al., 2009; Muranishi et al., 2012; Pak et al., 2014; Zhang et al., 2000) and play a central role in eye development. Disruption of Rx leads to small or absent eyes in mouse (Bailey et al., 2004; Mathers et al., 1997; Medina-Martinez et al., 2009; Muranishi et al., 2012; Zhang et al., 2000) and is associated with anophthalmia in humans (Voronina et al., 2004). In zebrafish, loss of function of Rx3, including mutation in the zebrafish rx3 gene (chk mutant), disrupts eye morphogenesis (Kennedy et al., 2004; Loosli et al., 2003; Stigloher et al., 2006): retinal progenitors are specified, but remain trapped in the lateral wall of the diencephalon, failing to undergo appropriate migration (Rembold et al., 2006) and differentiation (Stigloher et al., 2006).

In addition to its well-documented role in eye formation, Rx/rx3 governs hypothalamic development. Rx-null mice show variable penetrance, but all display abnormalities in the ventral hypothalamus (Mathers et al., 1997; Medina-Martinez et al., 2009; Zhang et al., 2000). Lineage-tracing studies demonstrate that Rx progenitors give rise to Sf1+ VMN and Pomc+ Arc tuberal neurons, and targeted ablation of Rx in a subset of VMN progenitors leads to a fate switch from an Sf1+ VMN identity to a Dlx2+ dorsomedial nucleus (DMN) identity (Lu et al., 2013). These studies suggest that Rx functions in progenitor cells to cell-autonomously select Sf1+ VMN and Pomc+ Arc identities. In zebrafish, chk mutants and rx3 morphants similarly show reduced...
numbers of pevTub pomc+ neurons and additionally decreased NPO avp+ (formerly vt, arginine vasotocin) neurons (Dickmeis et al., 2007; Tessmar-Raible et al., 2007), although currently the underlying mechanism is unclear. These studies, together, raise the possibility that Rx/rx3 plays a widespread role in the differentiation of tuberal and anterior/NPO hypothalamic neurons.

In mice, expression of the secreted signalling ligand Shh overlaps with that of Rx (Shimogori et al., 2010) and conditional ablation of Shh from the anterior-basal hypothalamus results in phenotypes that resemble the loss of Rx, including a reduction/loss of Avp+ PVN and POMC+ Arc neurons (Shimogori et al., 2010; Szabo et al., 2009). As yet, however, the link between Shh and Rx/Rx3 remains unclear and the mechanisms that operate downstream of Shh and Rx/Rx3 to govern hypothalamic differentiation are unresolved.

Here, we analyse rx3 and shh expression and function in the developing zebrafish hypothalamus. Analysis of chkh mutant and rx3 morphant fish, together with 5-ethyl-2'-deoxyuridine (EdU) pulse-chase experiments, show that Rx3 is required for a switch in progenitor domain identity, and for the survival and anisotropic growth of tuberal/anterior progenitors, including their progression to rx3+shh+ AR cells and to pomc+, fflb (nr5a1a)+ and opb+ Th1 (Th) tuberal/anterior fates. Timed delivery of cyclopamine or SAG reveals that Shh signalling governs these processes via dual control of rx3 expression, inducing then downregulating it. We demonstrate that rx3 downregulation, mediated by Shh signalling, is an essential component of Rx3 function: failure to downregulate rx3 leads to the failure of anisotropic growth, loss of the shh+rx3+ AR and failure of tuberal/anterior cell differentiation. Together, our studies reveal a mechanism that elaborates early patterning around the hypothalamic ventricle by the selective growth of distinct progenitor cells.

RESULTS
rx3 expression in third ventricle cells
Previous studies have described zebrafish rx3 expression (Bielen and Houart, 2012; Cavodeassi et al., 2013; Chuang et al., 2009; Kennedy et al., 2004; Loosli et al., 2003; Stigloher et al., 2006) but have not performed a detailed analysis in the 2- to 3-day embryo. Neurons in the hypothalamus, including pomc+ and avp+ neurons that are decreased/lost in the absence of rx3 (Dickmeis et al., 2007; Tessmar-Raible et al., 2007) begin to differentiate over the first 2-3 days of development (Liu et al., 2003; Dickmeis et al., 2007; Tessmar-Raible et al., 2007) and we therefore focused on this period. At 55 hours post-fertilization (hpf), rx3 is detected in three adjacent zones in the hypothalamus (Fig. 1A-B'). In keeping with mouse nomenclature (Lu et al., 2013), we term these zones I, II and III, characterized by the thin strip of weakly rx3-positive [rx3(weak+)] cells in zone II. Sections show that at its rostral limit, in zone I, rx3 is expressed in neuroepithelial-like cells around the AR and LR of the third ventricle (Fig. 1C,D) but is excluded from the AR tips (Fig. 1C 'D', arrowheads). In zone II, rx3 labels cells that closely line the AR/LR, again excluded from the AR tips (Fig. 1E,E', arrowheads). In zone III, rx3 marks neuroepithelial-like cells around the third ventricle, which in this region (between anterior and posterior recesses, see Fig. 1A,B') is small (Fig. 1F,F'). At 30 hpf, the entire third ventricle is small and lined throughout by rx3+ neuroepithelial-like cells (Fig. 1G-I). Thus, the well-defined recesses of the third ventricle, and characteristic rx3+ profiles, develop over 30-55 hpf.

Tuberal/anterior hypothalamus elongates from proliferating rx3+ progenitors
To determine the position of rx3+ cells relative to other hypothalamic regions, we compared rx3 expression with that of emx2 and fgf3, which mark the posterior, ventro-tuberal and dorso-anterior hypothalamus (Herzog et al., 2004; Kapsimali et al., 2004; Liu et al., 2013; Mathieu et al., 2002), and with the position of the adenohypophysis and the diencephalic-telencephalic junction (DTJ), which are morphologically distinct landmarks. Over 30-55 hpf, rx3 expression is rostral and largely complementary to emx2, and is sandwiched between ventro-tuberal and dorso-anterior fgf3+ cells (Fig. 2A-H, schematics in 2O), and in zone III it overlies the adenohypophysis. This suggests that throughout 30-55 hpf rx3 demarcates cells at the boundary of the posterior and tuberal/anterior hypothalamus.

Prior to 30 hpf, rx3 is expressed in progenitor cells (Bielen and Houart, 2012; Cavodeassi et al., 2013; Loosli et al., 2003; Rembold et al., 2006; Stigloher et al., 2006) and the third ventricle is known to harbour cycling cells (Bosco et al., 2013; Lee et al., 2006; Wang et al., 2009, 2012; Wullimann et al., 1999). To address directly whether 30 hpf rx3+ cells proliferate, we pulsed fish with EdU, culled immediately, and analysed sections for EdU and rx3 expression (Fig. 2I). At 30 hpf, 77% EdU+ cells are rx3+ and the remainder immediately abut rx3+ cells (Fig. 2I', n=110 cells, 4 embryos). Co-analysis of alternate sections with EdU and phosphorylated histone H3 antibody (phosH3) shows that cells in S phase progress to M phase (Fig. 2J'). Analysis of control embryos with phosH3 and rx3 confirms that the majority of cycling cells at 30 hpf are rx3+ (68% phosH3+ cells co-express rx3; 32% phosH3+ cells abut rx3+ cells; Fig. 2K,N; n=76 cells, 4 embryos). Whole-mount views of embryos double-labelled with rx3 and phosH3 suggests that by 55 hpf, fewer cycling cells are rx3+ (Fig. 2L,L'). Sections confirm this, showing that at 55 hpf 35% cycling cells are rx3+, 28% abut rx3+ cells but 38% are now detected in the rx3+ recess tips (Fig. 2M,N; n=92 cells, 4 embryos).

Although expressed in proliferating cells, the rostro-caudal length of rx3 expression in zones I and III does not change over 30-55 hpf (Fig. 2A,E,O,P) indicating its dynamic regulation. Proliferation correlates, though, with rostro-caudal growth of the tuberal/anterior hypothalamus (Fig. 2A,E,O,P). Growth is greatest over 30-48 hpf (Fig. 2P), and is 2.5-fold greater than rostro-caudal growth of the posterior hypothalamus or the dorsal diencephalon over this period (Fig. 2Q). In summary, the tuberal/anterior hypothalamus shows anisotropic growth over 30-55 hpf, driven from proliferating rx3+ cells and their immediate neighbours.

Development of rx3+shh+ AR and tuberal/anterior immature neurons
We next characterized the growing tuberal/anterior hypothalamus. At 30 hpf, shh is detected uniformly in the hypothalamus (Fig. 3A,A'): double-fluorescence in situ hybridization (FISH) analysis reveals extensive co-expression with rx3 (Fig. 3D,D', yellow arrowheads). rx3+shh+ cells are bound rostrally and ventrally by rx3+shh+ cells (Fig. 3D', red arrowheads) and caudally/dorsally by shh- cells (Fig. 3D', green arrowhead). In the co-expressing region, rx3 is strongest dorso-caudally (Fig. 3D'). Similar expression domains are detected at 55 hpf (Fig. 3B,E) but a novel shh+rx3+ domain now projects in the tuberal/anterior hypothalamus (Fig. 3B,E,F,F', white arrowheads). This domain appears to be composed of cells that have downregulated rx3, resulting in the characteristic zone II, but is significantly (1.5-fold) longer at 55 hpf compared with 30 hpf (Fig. 3D,F, white arrows). Analysis of sections shows that in this domain, shh is restricted to cells that line the AR/LR (Fig. 3F') and shows that shh+rx3+ cells define the AR tips (Fig. 3F', arrowheads; Fig. S1A,A',C, red arrowheads). Our data show that zone II is characterised by shh+ AR cells, and, together with our previous data,
suggests that AR tip cells are shh⁺rx3⁻ progenitors that derive from adjacent rx3⁺shh⁺ progenitors.

In zebrafish, immature tuberal/anterior hypothalamic neurons can be characterized through expression of the transcription factor otpb (Eaton and Glasgow, 2007; Löh et al., 2009; Herget et al., 2014; Manoli and Driever, 2014), the nuclear receptor Nr5a1/Sf1 orthologue ff1b (Kuo et al., 2005) and the precursor polypeptide pome (Liu et al., 2003; Herzog et al., 2004; Dickmeis et al., 2007; Tessmar-Raible et al., 2007; Manoli and Driever, 2014). At 30 hpf, otpb is detected in the posterior hypothalamus and at the DTJ (Fig. 3G,G’) but by 55 hpf additional otpb⁺ cells are detected in the tuberal and anterior hypothalamus (Fig. 3H,H’, white arrowheads; see Eaton and Glasgow, 2007) adjacent to the shh⁺ AR (Fig. 3I). Ventral views show that otpb⁺ cells in the tuberal and anterior hypothalamus are periventricular, suggesting they are immature neurons (see Fig. 4C’; Herget et al., 2014). ff1b expression is detected at 30 hpf (Fig. 3J,J’), and by 55 hpf is expressed broadly in the tuberal hypothalamus. Sections reveal that ff1b is expressed in shh⁺ AR cells and adjacent periventricular cells (Fig. 3K-K’).

pome⁺ cells cannot be detected in the 30 hpf hypothalamus.
Fig. 2. Anterior/tuberal hypothalamus elongates from rx3+ progenitors. (A-H′) Side views after single or double FISH at 30 hpf (A-D) and 55 hpf (E-H; E′-H′ show high-power views of boxed regions). Arrows in A,C,E,G show distances measured for growth comparisons. Arrowheads in E′,F′ indicate position of recesses (colour-coded as in Fig. 1A). (I-K) Maximum intensity projections of representative sections through 30 hpf embryos. I,J show serial adjacent sections; I′,J′ show single-channel views. Arrowheads show co-labelled (yellow) or single-labelled (green) cells. T-shaped white dotted lines indicate outline of AR and LR. (L,L′) Side views of 55 hpf embryo; L′ shows single-channel view. (M,M′) Representative single-plane views taken through zone II; M′ shows single-channel view. Yellow arrowheads show double-labelled cells; green arrowheads point to phosH3+, rx3− cells at recess tips. (N) Quantitative analyses of cycling cells at 30-55 hpf as indicated by phosH3 expression in rx3+ cells, rx3− cells or in cells adjacent (adj.) to rx3+ cells. (O) Schematic depicting rx3, fgf3 and emx2 expression, and change in length and axial orientation of hypothalamus. A 'bending' of the tuberal/anterior hypothalamus occurs over 30-55 hpf, relative to the rostro-caudal axis. Red arrows indicate length of dorsal diencephalon or length of emx2+ PH; white arrows indicate length of rx3+ territories; blue arrows indicate distance from DTJ to rx3+ zone III. (P) Length from DTJ to rostral tip of rx3+ zone III (n=5 embryos each at 30, 40, 48, 55 hpf). (Q) Tuberal/anterior hypothalamus grows approx. 2.5-fold more than dorsal diencephalon, emx2+ PH or ventral rx3+ zone III (n=10 each; P<0.0001). Dotted and dashed lines delineate ventral hypothalamus and T-shaped AR/LR (white), adenohypophysis (blue) and rx3-expressing domain (red). AH, anterior hypothalamus; dA, dorso-anterior; PH, posterior hypothalamus; TH, tuberal hypothalamus; vT, ventral tuberal. Scale bars: 50 µm.
but by 55 hpf are detected in the tuberal hypothalamus (Fig. 3N, white arrowheads). Together, our data show that anterior elongation correlates with the development and growth of the shh+rx3− AR and with the differentiation of otpb+, ff1b+ and pomc+ cells in the tuberal/anterior hypothalamus (schematized in Fig. 3O,P).

Fig. 3. Differentiation in the 30-55 hpf anterior/tuberal hypothalamus. (A-N) Side views (A,B,D,E,G-J,L-N), ventral views (C,F), sagittal (K) or transverse (K′,M′) sections of 30 hpf and 55 hpf embryos. A′-M′ show high-power views of boxed regions. In B′,E′,F', white arrowheads point to shh+AR cells; in H′, to otpb+ cells in the tuberal/anterior hypothalamus; in M′,N, to hypothalamic pomc+ cells. In D′,E′, arrowheads point to rx3+shh+ cells (yellow), rx3+ cells (red) or shh+ cells (green). (O,P) Schematics depicting expression domains at 30 hpf (O) or 55 hpf (P). AH, anterior hypothalamus; PH, posterior hypothalamus; PO, preoptic hypothalamus; TH, tuberal hypothalamus. Scale bars: 50 µm.
Rx3 is required for shh+ AR and neuronal differentiation

We next addressed the requirement for Rx3 in development of the tuberal/anterior hypothalamus. Previous studies have shown that pomc+ and avp+ neurons are absent in embryos lacking rx3 (Dickmeis et al., 2007; Tessmar-Raible et al., 2007) but a more extensive characterization of other progenitor/differentiating cells has not yet been performed.

Analysis of 55 hpf chk embryos shows that the shh+ AR fails to develop in chk mutants (Fig. 4A-B″, white asterisks; note that posterior shh expression in the floor plate and basal plate appears...
to be unaltered). rx3 expression itself is markedly different in chk mutant embryos compared with siblings: zones I-III cannot be clearly resolved (Fig. 4A′,B′,G-H′).

The failure in development of the shh+ AR correlates with a failure in differentiation. Mutant embryos lack otpb+ cells in both the tuberal and anterior hypothalamus [Fig. 4C-D′, white arrowheads; note that otpb+ cells in the posterior hypothalamus and at the DT3 (green arrowheads) appear to be unaffected]. Previous studies suggest that the anterior otpb+ progenitors give rise to Group 2/3 Tyrosine hydroxylase (Th) dopaminergic neurons (Löhr et al., 2009); in keeping with this, mutant embryos lack Group 2/3 Th1+ neurons (Fig. 4E-F′, white arrowheads: note Group 4-6 Th1+ neurons are not eliminated). rx3 mutant embryos additionally lack pomc− cells (Fig. 4I-J′, white arrowheads) and fflb− cells (Fig. 4K-L′, white arrowheads) in the tuberal hypothalamus [note pomc− cells in the adenohipophysis (green arrowheads) are still detected]. Finally, fez1, a homeodomain (HD) gene that in mouse is regulated by Sfi1 (Kurrasch et al., 2007) and in fish regulates otpb (Blechman et al., 2007), is markedly reduced (Fig. 4M-N′, white arrowheads); at the same time, ectopic expression is detected in the telencephalon.

Rx3 represses dorsal and ventro-tuberal progenitors

We postulated that, as in mouse (Lu et al., 2013), Rx3 may switch the identity of other progenitor domains to select posterior tuberal/anterior progenitor fates, and that the absence of Rx3 will lead to alterations in progenitor domains/increased alternative fates.

The transcription factor nkx2.1 (previously known as nkx2.1a; Manoli and Driever, 2014), the homologue of which in mouse is required for tuberal neuronal differentiation (Correa et al., 2015; Kimura et al., 1996; Yee et al., 2009), shows subtle differences in expression in chk mutants at 25 hpf: two sets of nkx2.1+ cells in the forming tuberal/anterior hypothalamus (Fig. 5A,A′, blue arrowheads) cannot be detected (Fig. 5B,B′). By 55 hpf, this difference is pronounced: nkx2.1 is reduced in the anterior hypothalamus and is not detected in the rostral tuberal hypothalamus [Fig. 5C,D; position of tuberal/anterior hypothalamus confirmed through double-labelling with shh (Fig. 5C′,D′)]. nkx2.1 in the caudal tuberal, posterior hypothalamus and posterior tuberculum appears to be unchanged.

Previous studies show that Nkx and Pax6 transcription factors exert cross-repressive interactions in the hypothalamus (Manoli and Driever, 2014), prompting us to examine expression of pax6. In control embryos, pax6 is confined to the thalamus/dorsal hypothalamus and abuts the dorsal-most boundary of rx3 (Fig. 5E,E′). In the absence of rx3, pax6 is detected ectopically in the tuberal/anterior hypothalamus within and rostral to rx3+ cells (Fig. 5F, red arrowheads; Fig. S2). Thus, the absence of rx3 leads to a ventral expansion of pax6+ progenitors.

Ectopic pax6+ domains do not extend throughout rx3 zone III (Fig. 5F, red dotted outline) raising the question of whether other progenitors are also affected by loss of rx3. The et5 transcription factor pea3 (etv4) is expressed in the hypothalamus at 30 hpf, and overlaps with rx3 zone III cells (Fig. S5G′,G″). pea3 is downregulated at 55 hpf in control embryos but expression persists in chk mutants (Fig. S5H,I). These results suggest that Rx3 normally suppresses both dorsal pax6+ and ventro-tuberal pea3+ progenitors (Fig. S5,K schematics) and predicts a widespread change in the profile of other progenitor markers in chk mutants. In support of this idea, asc11a and sox3 are not downregulated in zone II in chk mutant embryos, in contrast to their appearance in controls (Fig. S4A-H, white arrowheads).

In mouse, conditional ablation of Rx leads to a failure to select arcuate/VMN fates and, instead, additional Dlx2+ DMN cells form (Lu et al., 2013). To determine whether the increase in pea3 and pax6 expression results in an increase in ventro-tuberal and DMN-like cells, respectively, we examined the neurohypophyseal marker fgf3 and the DMN marker dlx1 (dlx1a). Both show slightly stronger expression in chk mutants (Fig. S4E-H), and the ventro-tuberal hypothalamus appears longer in chk mutants (Fig. S4A,C) suggesting that in the absence of Rx3, there is some expansion of ventral-tuberal and dorsal progenitors and their derivatives.

Rx3 is required for progenitor survival and anisotropic growth

The increase in fgf3 and dlx1 in chk mutants is, however, mild, suggesting that Rx3 may play a role other than switching progenitor fates. In sectioned embryos we had noticed an unusually disorganized accumulation of shh+ cells (Fig. 6A-C,G-I) suggesting that some ectopic progenitors may accumulate in the recess walls, rather than grow and progress to normal fates.

To examine this further, we compared proliferation and fate in control and rx3-null embryos. In comparison to controls, rx3-morphant and chk mutant embryos showed significantly more phosH3′ cells in the 55 hpf embryo (Fig. 6D,F-J′) that, in contrast to controls, were largely rx3+ or adjacent to rx3+ cells (Fig. 6L). To determine more specifically the fate of proliferating progenitors, we pulsed 30 hpf fish with EdU, chased to 55 hpf and, on serial adjacent sections, analysed whether EdU+ cells progressed to periventricular cells in the tuberal/anterior hypothalamus, were retained as rx3+ or shh− progenitors, or assumed other fates. In chk siblings, the majority (63%, n=156 cells, 6 embryos) of EdU+ cells were laterally oriented chains in the anterior (Fig. 6M) or tuberal (Fig. 6P) hypothalamus and were detected in or in the vicinity of fflb+ and pomc− cells (Fig. 6M,O,P). A minority (27%) were shh− rx3− anterior (Fig. 6N,O) or lateral (not shown) recess tip cells. No EdU+ rx3+ cells were detected in zones I or III (Fig. 6R; data not shown). By contrast, in chk mutant embryos, no EdU+ cells were detected in the region rostral to the adenohipophysis, i.e. the region that would form part of the anterior/tuberal hypothalamus (Fig. 6T-W). The majority (76%, n=165 cells, 6 embryos) of EdU labeling was detected in/adjacent to shh− (Fig. 6X) and rx3+ (Fig. 6Y) cells. EdU+ cells accumulated especially at the recess junctions and tips. No cleaved (c)Caspase+ cells were detected after the 25 h chase period, but after a 5 h chase, cCaspase+ cells, including EdU+cCaspase+ cells were detected in chk mutants (Fig. 6Z). No cCaspase was detected in siblings (Fig. 6S).

These findings, together with our previous observations, suggest that rx3+ progenitors give rise to cells, including shh− AR tip cells, that grow anisotropically and give rise to anterior/tuberal cells. Additionally, these findings show that in the absence of Rx3 function, many progenitor cells accumulate in the recesses, where they either die, or fail to differentiate. Together, these observations point to a mechanism in which Rx3 selects tuberal/anterior progenitors and governs their survival and growth (Fig. 6 schematics).

Shh is an ‘on-off’ switch for rx3

Our findings demonstrate that Rx3 is upstream of that of shh in the tuberal/anterior hypothalamus. However, given the crucial role of Shh...
in induction and early patterning of the hypothalamus (Bedont et al., 2015; Burbridge et al., 2016; Pearson and Placzek, 2013; Blaess et al., 2015), we wished to test whether at earlier stages of hypothalamic development Shh is upstream of *rx3*, a possibility suggested by the observation that at epiboly stages, *shh* is expressed on midline cells, close to the early zone of *rx3* expression (Fig. S5A,B).

**Ptch1**, a Shh-receptor and ligand-dependent antagonist, is weakly detected in the forming tuberal/anterior hypothalamus at 30 hpf (Fig. 7A), but not detected when embryos are exposed to cyclopamine over 10-28 hpf (Fig. 7G). Similar observations were made with **ptch2** (not shown). At the same time, cyclopamine treatment results in a marked downregulation of *rx3* (Fig. 7B,H) mimicking the phenotype of slow muscle omitted (smu) mutant zebrafish that lack essential components of the Hh pathway (Fig. S5C,D). Together, these results suggest that Shh induces *rx3* in the early embryo.

By 55 hpf, strong **ptch1** expression is detected in zones I and III (Fig. 7C) with weaker expression in zone II (Fig. 7C). **ptch2** expression appears similar (not shown). To determine whether Shh influences *rx3* at this stage, we exposed embryos to cyclopamine...
over 28-55 hpf. This resulted in an effective inhibition of Shh signalling, as judged by *ptch1* downregulation (Fig. 7I) but led to a consistent increase in *rx3* expression (Fig. 7D,J). Increased *rx3* expression was accompanied by changes that appeared to phenocopy loss of *rx3*, notably a significant decrease in tuberal/anterior territory (Fig. 7D,J white lines and red arrows), a decrease in hypothalamic *pomc*+ cells (Fig. 7E,K,M), the loss of *ff1b* expression (Fig. 7F,L), a decrease in Th1+ Group2/3 neurons (Fig. S5E,F; note Groups 4-6 in the posterior hypothalamus are unaffected) and a failure to downregulate *sox3* in zone II (not shown). These observations suggest that Shh mediates *rx3* downregulation in zone II, and that this is essential for differentiation of tuberal/anterior hypothalamic progenitors.

This idea predicts that provision of Shh may be sufficient to rescue the phenotypic effects of *rx3* morphant embryos, once the effects of the morpholino begin to disappear. To test this, we attempted a ‘late rescue’, in which *rx3* morphant embryos were exposed to the small molecule Shh agonist SAG over 28-55 hpf. SAG was effective in restoring a normal pattern of Shh signalling in *rx3* morphant embryos, as judged by expression of *ptch1* (Fig. 7N,T). Furthermore, both the normal pattern of *nkh2.1* and the characteristic profile of *rx3* in zones I, II and III were restored (Fig. 7O,P,U,V). Both *pomc*+ and *ff1b*+ cells were restored in *rx3* morphant embryos in response to SAG administration (Fig. 7Q,R,W,X). Finally, cellular homeostasis was restored: the enhanced numbers of phosH3*+* cells in *rx3* morphants were reduced to normal, wild-type levels (Fig. 7S′,Y). This rescue is not seen when an early SAG-treated regime is used (10-28 hpf; not shown), or in *chk* mutant embryos treated with SAG over 28-55 hpf (Fig. S6), indicating that functional *Rx3* is required for the late rescue. Together, these results suggest that a Shh-rx3 ON and Shh-rx3 OFF feedback loop (Fig. 7Z) is essential for the development of the tuberal/anterior hypothalamus.

**DISCUSSION**

Here, we show that Rx3 function is required for morphogenesis of the tuberal/anterior hypothalamus and governs three aspects of cell behaviour: it re-specifies progenitor types to tuberal/anterior identities, promotes their survival and governs their anisotropic growth/migration. Shh coordinates tuberal/anterior progenitor selection and behaviour by acting as an on-off switch for *rx3*. Thus, a Shh-Rx3-Shh feed-forward/feedback loop generates tuberal/anterior progenitors that grow to expand the surface area of the third ventricle and diversify the neuronal subtypes that differentiate around it.

**Rx3 selects tuberal/anterior hypothalamic progenitors**

Our studies confirm that Rx3 function is not required for induction or initial hypothalamic patterning (Kennedy et al., 2004), but show that it is essential to elaborate patterning. Our data suggest that Rx3 autonomously selects *nkh2.1*+ tuberal/anterior progenitors that grow anisotropically. In *chk* mutant embryos, *pax6a* expands ventrally into *rx3*+ progenitors, a phenotype detected as early as 19 hpf (Loosli et al., 2003). The ventral expansion of *pax6a* mimics the phenotype of *nkh2.1*/*nkh2.4a/nkh2.4b-null embryos (Manoli and Driever, 2014) and suggests that Rx3 re-specifies progenitors that would otherwise assume a dorsal hypothalamic or pre-thalamic identity.

At the same time, Rx3 represses *pea3*. In wild-type animals, *pea3* overlaps with the ventral-most domain of *rx3* expression at 30 hpf, but is downregulated by 55 hpf. In *chk* mutant fish, *pea3* expression persists. Although we have not performed double FISH with *pea3*
and pax6 in chk mutant fish, their expression patterns appear to be complementary. This suggests that Rx3 operates as a switch in at least two separate progenitor populations and provides a prosaic interpretation for the existence of two domains: the dorsal rx3+shh and ventral rx3–shh domains.

Our studies reveal that Rx3 promotes alternative fates in progenitor cells. Its loss leads to one of three outcomes: to undergo apoptosis or to be retained as a proliferating cell held in the wall of the ventricle (novel outcomes), or to initiate alternative adjacent differentiation programmes – after pulse-chase, some EdU+ cells are detected in periventricular regions in chk mutants where it is likely that they contribute to nkk2.1+pea3+ progenitors and hence fgf-expressing neurohypophysis ventrally, and to dlx1+ cells dorsally. dlx1+ cells are likely to be immature DMN-like neurons and notably, somatostatin neurons persist in chk mutants (Dickmeis et al., 2007). Together, our studies suggest that Rx3 selects tuberal/anterior neuronal progenitors and limits both ventro-tuberal neurohypophysial and DMN-like progenitors.

In addition to promoting cell survival, Rx3 regulates cellular homeostasis in the tuberal/anterior hypothalamus, orchestrating a balance of proliferation and differentiation. We surmise that the increased proliferation seen in the absence of Rx3 reflects changes in Wnt or Fibroblast growth factor signalling, both of which are upregulated in chk mutants (Stugloher et al., 2006; Yin et al., 2014; this study). fgf3, in particular, normally abuts neuroepithelial-like rx3+shh+ cells in both zones I and III and is upregulated in rx3+ mutants. Potentially, the driving force for proliferation resides in rx3+shh+ cells in zones I and III that progress to rx3+shh+ cells in zone II.

Previous reports have shown that Rx3 is required for retinal fate selection and that telencephalic fates are expanded in its absence (Bielen and Houart, 2012; Cavodeassi et al., 2013). Our studies likewise show changes in the telencephalon/eye territory: fezf1 is upregulated in rx3 mutants, and both shh and nkk2.1 in the telencephalon/tuberal/anterior area are greatly reduced. Together, these studies suggest that Rx3 selects fate in cells of distinct origins: anterior telencephalic and posterior diencephalic. Importantly, not all hypothalamic cells alter their identity in the absence of Rx3: the posterior hypothalamus expresses nkk2.1, shh and otpb as normal, the rostral-most hypothalamic expresses otpb and nkk2.1 and the tuberal hypothalamus expresses nkk2.1, pea3 and fgf3, emphasising the fact that Rx3 elaborates, rather than initiates, hypothalamic patterning.

**Shh is an on-off switch for rx3**

Our study shows that Shh is required for both the induction of rx3 and the progression of rx3+ to rx3+shh+ progenitors and demonstrates that both steps are required for tuberal/anterior hypothalamic neurogenesis. Downregulation of Shh signalling over 10-30 hpf leads to an almost complete loss of rx3 expression. By contrast, downregulation over 30-55 hpf leads to sustained rx3 in zone II and a phenotype that is highly similar to that of chk mutants: sox3 is not downregulated in zone II, the shh′rx3′ AR does not form, the tuberal/anterior hypothalamus is short and its resident neurons do not differentiate. Importantly, the Shh agonist SAG can restore normal patterns of proliferating progenitors and neuronal differentiation in late rx3 morphants. The most likely interpretation of these findings is that Shh-mediated rx3 upregulation is required to select tuberal/anterior progenitors but that Shh-mediated rx3 inhibition is required for these to realise their differentiation programme(s). Future studies are needed to establish whether the downregulation of sox3, nkk2.1, ascl1 and ptch1 that...
we observe in wild-type but not chk mutant fish are similarly required for progression of tuberal/anterior progenitors. We predict that the downregulation of pach1, in particular, supports Shh active signalling from zone II cells and contributes to development of the shh+ AR. The intricate regulation of induction and cessation of Shh signalling in sets of neighbouring cells is emerging as a common theme within the CNS (Briscoe and Therond, 2013) and provides the opportunity to drive expansion of territories and build increasingly complex arrays of neurons.

In summary, our studies suggest that Shh plays a dual role in rx3 regulation, inducing, then repressing it, and are consistent with a model in which Shh deriving from AR cells, feeds back to rx3+ progenitors to promote their further differentiation.

Origins of hypothalamic neurons
Our studies show that the zebrafish tuberal hypothalamus includes regions analogous to the mouse Arc and VMN. Our EdU pulse-labelling studies suggest that shh+ AR cells and differentiating ff1b+ and pomc+ neurons derive from rx3+ cells. After a 25 h chase, we detect strings of EdU+ cells, presumably of clonal origin, extending medio-laterally from the shh+ AR tips to pomc+ and ff1b+ regions, favouring the idea that forming neurons derive from rx3+shh+ progenitors via rx3+shh+ progenitors. In mouse, Rax+ cells give rise to Pomc+ and Sf1+ neurons (Liu et al., 2013). Other mouse studies show that Shh+ hypothalamic cells give rise to tuberal neurons (Alvarez-Bolado et al., 2012), and that Shh ablation in hypothalamic cells leads to the loss of Pomc and Sf1 (Shimogori et al., 2010) and a reduction in hypothalamic territory (Alvarez-Bolado et al., 2012; Zhao et al., 2012). These studies, together with observations that loss of Ncx2.1 results in loss of tuberal hypothalamic neurons (Correa et al., 2015; Kimura et al., 1996; Yee et al., 2009), disruptions to the infundibulum and a reduction in the size of the third ventricle (Kimura et al., 1996), suggest a conserved differentiation route of pomc+ and ff1b/sf1 immature neurons and the tuberal hypothalamus from zebrafish to mouse.

In zone I, rx3 is expressed in the anterior hypothalamus, in a region that may be equivalent to the anterior-dorsal domain reported in mouse (Shimogori et al., 2010). Our work, together with previous studies, suggests that here, Rx3 plays a role in a conserved differentiation pathway for avp+ and Group2/3 Th1+ neurons. avp+ and Group 2/3 Th1+ neurons localize within a discrete subregion of hypothalamic otp expression (Löhr et al., 2009; Herget et al., 2014; Herget and Ryu, 2015) and in fish, as in mouse, otp genes are required for the differentiation of neurons that express avp and Th (Acampora et al., 1999; Löhr et al., 2009; Fernandez et al., 2013). avp+ neurons fail to differentiate in the absence of rx3 (Tessmar-Raible et al., 2007) and we now show a specific loss of an otpb+ subset and Group 2/3 Th1+ neurons. This suggests that Rx3 governs a subset of otpb+ progenitors in the anterior hypothalamus that will give rise to avp+ and Group 2/3 Th1+ neurons. We have not yet investigated whether this otpb+ progenitor subset are dependent on Shh. However, in mouse, conditional deletion of hypothalamic Shh leads to a reduction in Otp expression and Avp+ neurons (Szabo et al., 2009) as well as a loss of Sim1 in the PVN (Shimogori et al., 2010), suggesting that the Shh-Rx3-Shh pathway that governs pomc+ and ff1b+ cell fates may likewise govern avp+ and Group 2/3 Th1+ fates. A previous study has highlighted Sim1 and Otf as core components of a conserved transcriptional network that specifies neuroendocrine as well as A11-related hypothalamic dopaminergic neurons (Löhr et al., 2009), suggesting that Rx3 may be intimately linked to this pathway. Notably, because other NPO neurons, including oxytocin+ (previously known as isotocin) neurons are not affected by loss of Rx3, our data suggest that neurons that make up the NPO derive from discrete lineages. Our work adds to a growing body of evidence that directed cell migrations play a pivotal role in ventral forebrain/hypothalamic morphogenesis (Varga et al., 1999; Cavodeassi et al., 2013 and see Pearson and Placzek, 2013). We do not know the mechanisms that operate downstream of Rx3 to govern appropriate migration, but Eph/Ephrin signalling, expression of Fgf and Netrin, all of which govern cell adhesion and migration of neural cells, are disrupted in chk mutant embryos (Cavodeassi et al., 2013; Yin et al., 2014, this study) and could contribute.

In conclusion, our study suggests a mechanism by which Shh elaborates patterning in the hypothalamus. Previous reports suggest that Shh patterns the early hypothalamus in many vertebrates, establishing early progenitor domains (reviewed by Pearson and Placzek, 2013; Blaess et al., 2015). Our study shows that in zebrafish, Shh elaborates early patterning by switching progenitor domain identity, and promoting the survival and anisotropic growth of the new progenitor cells. Recent studies in the developing spinal cord show that the coordination of growth and specification can elaborate patterning in an expanding tissue, if molecularly distinct neural progenitor domains undergo differential rates of differentiation (Kicheva et al., 2014), raising the possibility that Shh may govern differentiation rates in the tuberal/anterior hypothalamus. Studies in mice that reveal similarities in the phenotypes of embryos in which Shh or Rax are conditionally ablated raise the possibility that features of the mechanism that we describe here may be conserved in other vertebrates.

Finally, the Shh-Rx3-Shh loop that we describe provides a means to maintain a dynamic balance between proliferating and differentiating cells. Studies in mice show that at least a subset of Rax+ cells persist into adulthood as stem cells (Miranda-Angulo et al., 2014) that can direct hypothalamic neurogenesis even in postnatal life. The exquisite regulation of Shh, Fgf and Wnt signalling, via Rx3, is likely to hold the key to a better understanding of hypothalamic neurogenesis throughout life and support a better understanding of complex human pathological conditions and dysfunctional behaviours that are underlain by tuberal/anterior hypothalamic cells and circuits.

MATERIALS AND METHODS

Animals
Zebrfish were staged according to Kimmel et al. (1995). chkα-29 fish were kindly provided by Dr Brendan Kennedy (University College Dublin, Ireland).

Nomenclature
We use the terms preoptic, anterior, tuberal and posterior to define the rostro-caudal domains of the hypothalamus. The region we define as anterior may overlap with the region that is conventionally termed the NPO (see Discussion).

In situ hybridization
Single and double in situ hybridization methods were adapted from Thisse and Thisse (2006) and Lauter et al. (2011) (details in supplementary Materials and Methods). Embryos were post-fixed in 4% paraformaldehyde and visualized by Olympus Nomarski or confocal microscopy. For cryostat sectioning, embryos were re-fixed and equilibrated in 30% sucrose, and 15-µm-thick serial adjacent sections cut. n=10–40 embryos for whole mounts; n=4–6 embryos for sections.

EdU analysis
Embryos were pulsed with 300 µM EdU for 1 h on ice, chased for 1, 5 or 25 h, then processed for cryostat sectioning and double EdU/in situ
hybridization analysis (details in supplementary Materials and Methods) using the Click-iT EdU Alexa Fluor 488 Imaging Kit (Fisher Scientific).

**Immunohistochemistry**

Anti-phosphH3 (06-570, Millipore), anti-cleaved Caspase (9661, Cell Signaling Technology) and anti-Thl (22941, Immunostar) were used at 1:1000. Fixed embryos or sections were processed according to Liu et al. (2013) and mounted in VectaShield.

**Length measurements**

Length was determined through measurements of images, where in situ patterns could be detected relative to morphological landmarks (diencephalic-telencephalic junction, optic commissure, lateral ventricle, posterior hypothalamus and adenohypophysis). For each experiment, length was normalized to the average length of age-matched sibling controls.

**Cell quantification**

phosphH3$^+$ and EdU$^+$ cell numbers were obtained through counts in serial adjacent sections through individual hypothalami using in situ patterns against morphological landmarks (above) to determine relative position. For chk mutants, section position was determined relative to unaffected posterior hypothalums.

**Image acquisition**

Differential interference contrast or fluorescence images were acquired using Olympus BX60 Zeiss Confocal LSM510 Meta or Olympus Confocal microscopes. Data was processed with Adobe Photoshop CS3/Adobe Illustrator CS.

**Statistical analysis**

Statistical analyses were performed using Prism 5. Each data value sampled was tested for Gaussian distribution prior to unpaired t-test by performing baseline subtraction of the two datasets and analysed using the D’Agostino–Pearson omnibus normality test.

**Cyclopamine treatment**

Cyclopamine (in ethanol) was used at 50 μM, optimised on the basis of ptc1 downregulation (20, 50, 100, 120 μM tested). Cyclopamine or ethanol were added to dechorionated embryos, which were kept in the dark.

**SAG treatment**

SAG (Millipore-EMD chemicals) in DMSO was used at 10 μM, optimised on basis of ptc1 upregulation (2, 5, 8, 10 μM tested). SAG or DMSO was added to de-chlorionated embryos in E3 medium, and embryos were kept in the dark.

**Morpholino**

Morpholinos [0.25 mM rxl 3 ATG (targets TSS) and 0.15 mM rxl 3 E212 (targets splice site)] (GeneTools, LLC) (Tessmar-Raible et al., 2007) were injected into one-cell embryos and morphants were selected on the basis of Rx3 activity.

**References**


**Supplementary information**

http://dev.biologists.org/lookup/doi/10.1242/dev.138305.supplemental


