Differential requirements for neurogenin 3 in the development of POMC and NPY neurons in the hypothalamus

Michelle Pelling [a], Neal Anthwal [a], David McNay [a,1], Gerard Gradwohl [b], Andrew B. Leiter [c], Francois Guillemot [d], Siew-Lan Ang [a,*]

[a] *Division of Developmental Neurobiology, MRC National Institute for Medical Research, The Ridgeway, London, NW7 1AA, UK*
[b] *Gerard Gradwohl, IGBMC, CNRS, INSERM, ULP, F-67404, Illkirch, C.U. de Strasbourg, France*
[c] *Department of Cellular and Molecular Physiology, Tufts-New England Medical School, Boston, MA, USA*
[d] *Division of Molecular Neurobiology, MRC National Institute for Medical Research, The Ridgeway, London, NW7 1AA, UK*

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ABSTRACT

The neuroendocrine hypothalamus regulates a spectrum of essential biological processes and underlies a range of diseases from growth failure to obesity. While the exploration of hypothalamic function has progressed well, knowledge of hypothalamic development is poor. In particular, very little is known about the processes underlying the genesis and specification of the neurons in the arcuate and ventromedial nuclei. Recent studies demonstrate that the proneural basic helix-loop-helix transcription factor Mash1 is required for neurogenesis and neuronal subtype specification in the ventral hypothalamus. We demonstrate here that Ngn3, another basic helix-loop-helix transcription factor, is expressed in mitotic progenitors in the arcuate and ventromedial hypothalamic regions of mouse embryos from embryonic days 9.5–17.5. Genetic fate mapping and loss of function studies in mice demonstrate that Ngn3+ progenitors contribute to subsets of POMC, NPY, TH and SF1 neurons and is required for the specification of these neuronal subtypes in the ventral hypothalamus. Interestingly, while Ngn3 promotes the development of arcuate POMC and ventromedial SF1 neurons, it inhibits the development of NPY and TH neurons in the arcuate nuclei. Given the opposing roles of POMC and NPY neurons in regulating food intake, these results indicate that Ngn3 plays a central role in the generation of neuronal populations controlling energy homeostasis in mice.

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Introduction

The mammalian hypothalamus is involved in regulating multiple physiological functions, which include growth and development, immune system activity, thermoregulation, food intake, fluid homeostasis, sleep regulation, as well as reproductive and maternal behaviours (Krononberg et al., 2007; Michaud, 2001). The rodent hypothalamus has a complex structure composed of multiple nuclei corresponding to dense groups of neuronal cell bodies. These neurons project axons to numerous regions of the CNS in addition to producing neuropeptide hormones (Swanson and Sawchenko, 1983). One group of neuroendocrine neurons, located in the arcuate nucleus (ARC) and ventromedial region (VMH) of the hypothalamus, do not release neuropeptides into the general circulation, but rather respond to peripheral hormones in order to regulate energy balance. In brief, energy related signals such as leptin are integrated by responsive first order neurons such as the anorexic pro-opoiomelanocortin (POMC)/

cocaine- and amphetamine-regulated transcript (CART) neurons and orexic neuropeptide Y (NPY)/Agouti-related peptide (AgRP) neurons, and relayed to second order melanocrotin-4 receptor (MC4R) expressing neurons in the PVN and the lateral hypothalamic area. The opposing effects of POMC and NPY neurons results in a negative feedback loop that maintains energy balance within physiological parameters (reviewed in Barsh and Schwartz, 2002; `Broberger, 2005; Morton et al., 2006; Srinivas et al., 2001). In addition to the POMC and NPY neurons of the ARC, steriodiogenic factor 1 (SF1) expressing neurons of the VMH are central regulators of energy balance in response to leptin and deletion of leptin receptor in SF1-expressing neurons results in increase body weight and susceptibility to diet-induced obesity in mice (Dhillon et al., 2006).

Neurogenin1 (Ngn1), Ngn2 and Ngn3 are basic-helix-loop-helix (bHLH) proteins, which together define a novel subfamily of *atonal*-related genes (Gradwohl et al., 1996; Ma et al., 1996; Sommer et al., 1996). All three genes are expressed in the hypothalamus in both zebrafish (Wang et al., 2001) and mice (McNay et al., 2006; Ravassard et al., 1997; Sommer et al., 1996). Ngn1 and Ngn2 function as proneural genes in the cranial sensory ganglia and spinal cord and thus promote both neurogenesis and notch-delta mediated lateral inhibition (reviewed in Bertrand et al., 2002). Ngn2 also regulate neuronal subtype
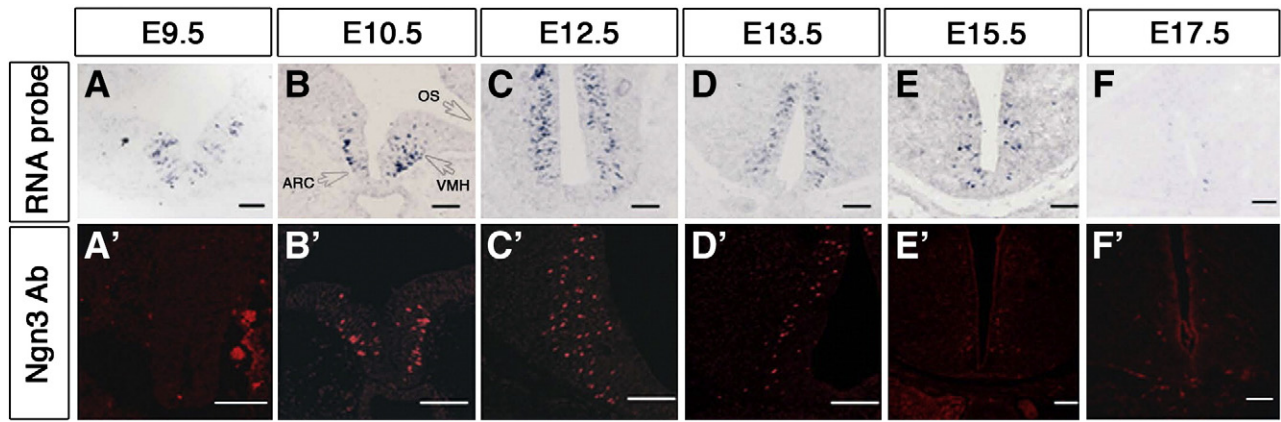
Fig. 1. Ngn3 mRNA and protein expression pattern in the ventral hypothalamus. (A–F') Coronal sections of mouse embryos. Ngn3 transcripts and protein are similarly expressed in the lateral margins of the ventricular zone surrounding the third ventricle of the VMH and ARC in the hypothalamus from E9.5 to E17.5. Scale bar corresponds to 100 μm. Abbreviations: OS, optic stalk.

identity in the cerebral cortex, acting to promote glutaminergic cell fate (Fode et al., 2000).

Loss and gain of function studies of Ngn3 in mice suggest that Ngn3 acts as a genetic switch that specifies an endocrine cell fate in pluripotent pancreatic progenitors (Apelqvist et al., 1999; Grapin-Botton et al., 2001; Schwitzgebel et al., 2000); however, nothing is known about its role in neuronal lineages. Ngn3 is also required for endocrine cell fate specification in multipotent intestinal progenitor cells (Jenny et al., 2002; Lee et al., 2002). We therefore determined whether Ngn3 might have similar roles in regulating neurogenesis and neuronal subtype specification in the hypothalamus. Firstly, we demonstrate that Ngn3 is expressed in mitotic progenitors in the ARC/VMH regions of the hypothalamus. Secondly, genetic fate mapping studies provided evidence that Ngn3 progenitors contribute to subsets of arcuate *TH,* POMC, NPY neurons and ventromedial *SF1* neurons. Thirdly, analysis of *Ngn3*$^{-/-}$ mice demonstrates that Ngn3 is required for proper development of all these neuronal subtypes. Interestingly, while Ngn3 promotes the development of arcuate POMC and ventromedial SF1 neurons, it inhibits the development of NPY and TH neurons in the arcuate nuclei. Since POMC and NPY have opposing regulatory functions in regulating food intake, the differential requirements for Ngn3 in the development of these neurons suggests that Ngn3 likely plays a critical role in regulating energy homeostasis in mammals.

Materials and methods

Generation and genotyping of mutant embryos and animals

Ngn3$^{+/-}$ mice, and *Ngn3Cre* mice were generated and genotyped as previously described (Gradwohl et al., 2000; Schonhoff et al., 2004). The *Ngn3* mutant animals were kept as heterozygous stocks and intercrossed to obtain *Ngn3*$^{-/-}$ mutants at different embryonic stages. The *Ngn3Cre* heterozygous mice were crossed with *R26R*$^{YFP/YFP}$ reporter mice (Srinivas et al., 2001) and were kept as double heterozygous mice. Male *Ngn3Cre/+;R26R*$^{YFP/+}$ animals were crossed with *R26R*$^{YFP/YFP}$ females to obtain *Ngn3Cre/+;R26R*$^{YFP/+}$ or *Ngn3Cre/+;R26R*$^{YFP/YFP}$ embryos (referred to henceforth as *Ngn3;R26R*^{YFP} embryos) for the genetic fate mapping studies. At all times, animals were handled according to the Society of Neuroscience Policy on the Use of Animals in Neuroscience Research, as well as the European Communities Council Directive.

Immunohistochemistry and in situ hybridisation of brain sections

Embryos or dissected embryonic heads were fixed for 30 min (E10.5–E13.5), 1 h (E15.5), and overnight (E17.5) at 4 °C in 4% paraformaldehyde in PBS and cryoprotected with 30% sucrose in PBS, then embedded in OCT compound (VWR International, Poole, UK). The blocks were then cryosectioned on a cryostat at 10 μm for E10.5, 12 μm for E13.5 and E15.5, and 14 μm for E17.5 (CM3050S; Leica, Nussloch, Germany).
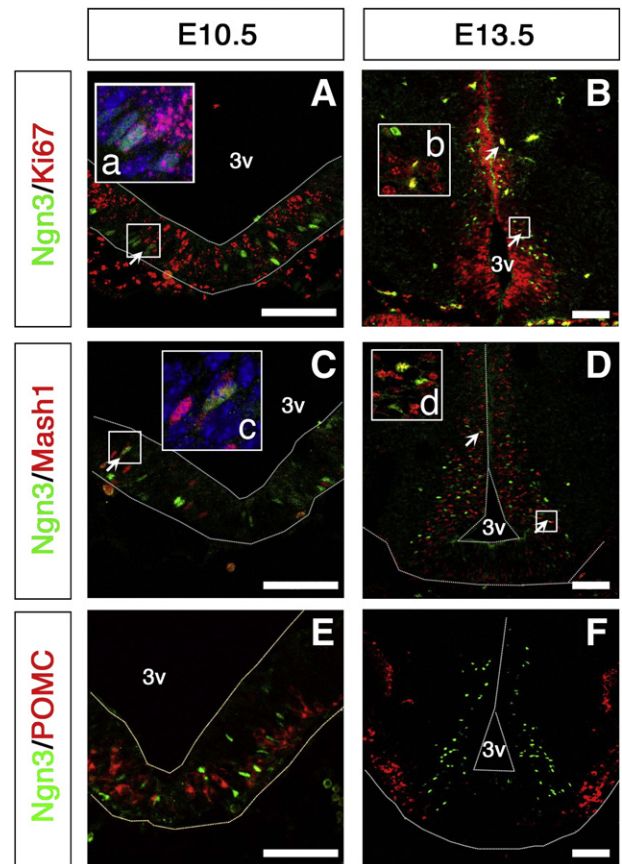

Fig. 2. Ngn3 is expressed in ventral hypothalamic progenitors. (A–F) Coronal sections of mouse embryos. (Inserts a–d) Higher magnification of the boxed region in A–D with nuclei counterstained with DAPI (blue). (A–D) Double immunofluorescent staining reveals that Ngn3 is co-expressed with mitotic markers, such as Ki67 (arrows in A, B and inserts a and b) and Mash1 (arrows in C, D and inserts c and d) at E10.5 and E13.5. (E, F) Ngn3 is not co-expressed with POMC in postmitotic ARC neurons at E10.5 (E) or E13.5 (F). The neural tissue is outlined by dotted lines in A and C–F. Scale bar corresponds to 75 μm. Abbreviations: 3v, third ventricle.
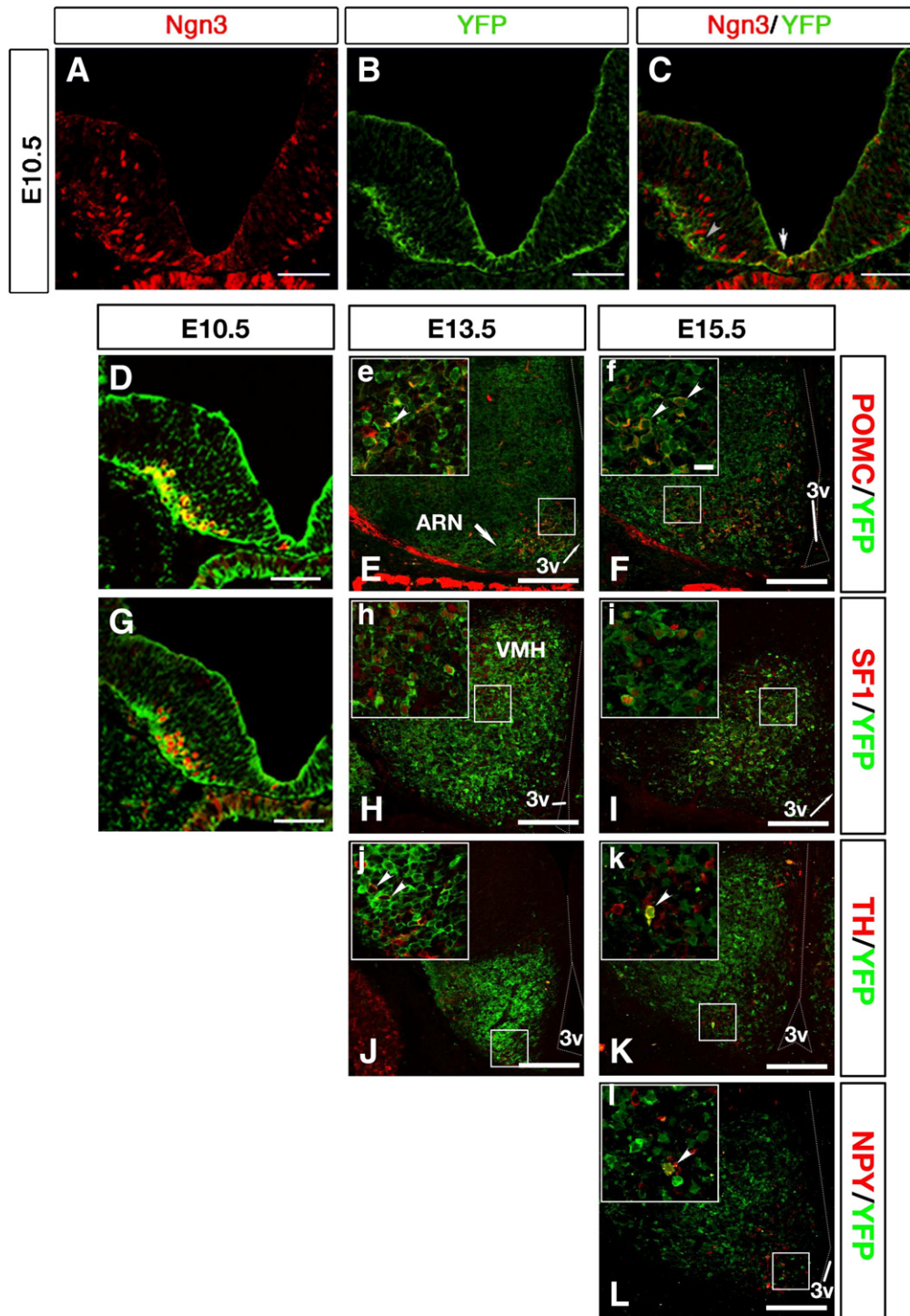
Fig. 3. Co-localization of Ngn3 and YFP in *Ngn3Cre;R26R^Yfp* embryos (A–C) and demonstration that Ngn3+ progenitors contribute to POMC, SF1, TH and NPY neurons in the ventral hypothalamus (D–L). (A–L) Coronal sections of mouse embryos. (A–C) Co-expression of YFP and Ngn3 in ventral hypothalamic cells (arrow) and postmitotic neurons (arrowhead) of *Ngn3Cre/+;R26RYFP* embryos. (D–L) Double immunofluorescent (arrow heads) staining showing co-expression of YFP with POMC (D–F), YFP with SF1 (G–I), YFP with TH (J, K) and YFP with NPY (L) in postmitotic neurons of the ventral hypothalamus in *Ngn3Cre/+;R26RYFP* embryos at various stages. Insets e, f, h–l show higher magnification of boxed region in the corresponding panels. Arrowheads point to double-labelled cells in these insets. The third ventricle (3v) is outlined by dotted lines. Scale bar corresponds to 100 μm (A–D, G), 150 μm (E, F, H–L) or 15 μm (e, f, h–l).

Section in situ hybridisations was performed as previously described (Vernay et al., 2005). The following antisense RNA probes have been used: *Mash1* (Guillemot and Joyner, 1993), *POMC* (Good et al., 1997), *Ngn3* (Gradwohl et al., 2000), *Npy* (Higuchi et al., 1988), *Nhlh2* (Good et al., 1997), *NeuroD* (Lee et al., 1995), *Scg10* (Stein et al., 1988), *Dll1* (Bettenhausen et al., 1995) and *Hes5* (Casarosa et al., 1999).

For immunohistochemistry, sections were incubated overnight at 4 °C with the appropriate primary antibody diluted in 0.1% TritonX100 and 1% BSA in PBS. Sections were then extensively washed in PBS and incubated for 2 h at room temperature with a secondary antibody conjugated with a fluorochrome. For double antibody labelling experiment, sections were washed and then incubated for 2 h at room
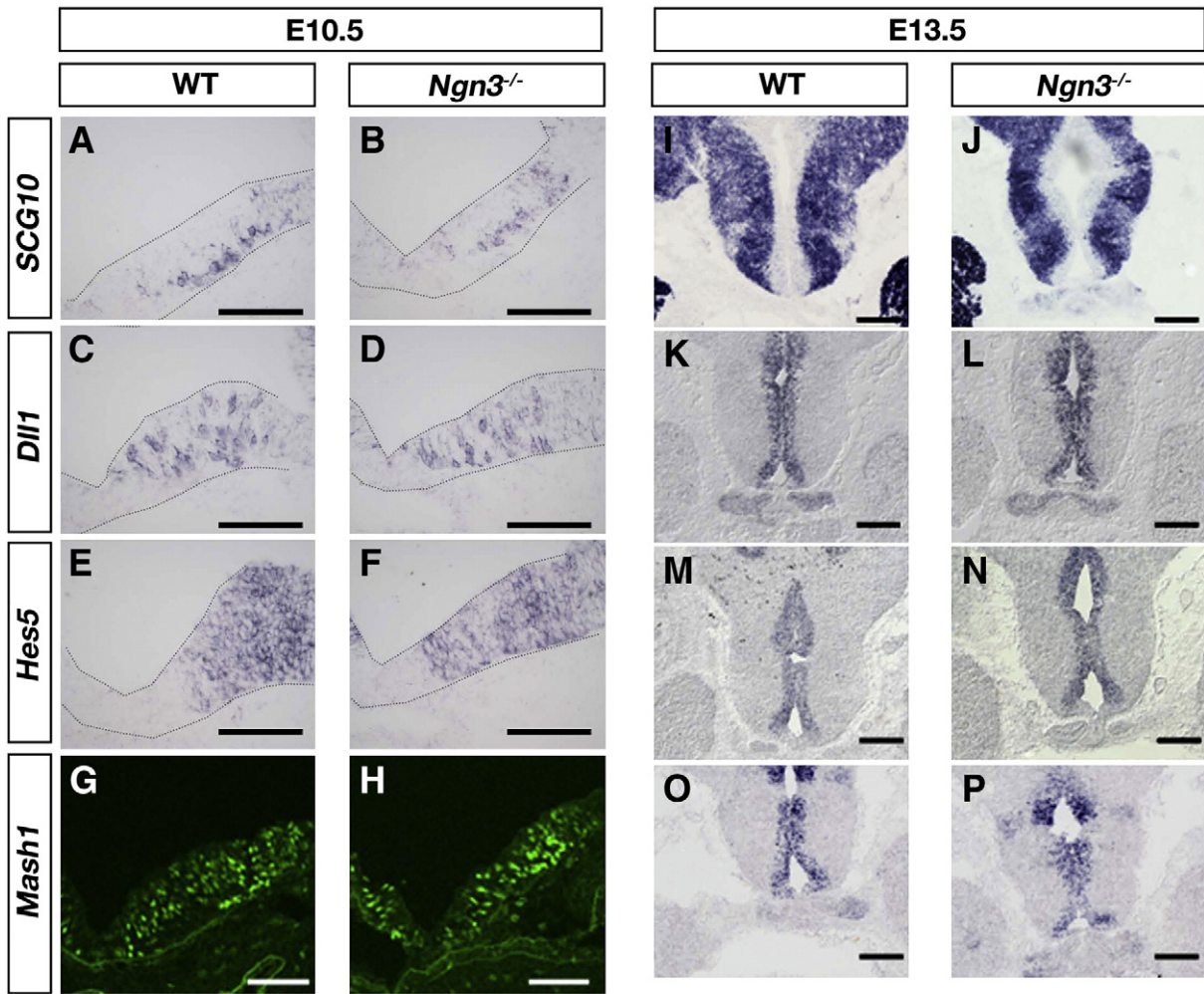
Fig. 4. Notch-mediated lateral inhibition, neurogenesis, proliferation and cell death are not affected in the *Ngn3* null mutants. (A–P) Coronal sections of mouse embryos. (A–F) No change in expression of *Scg10* (A, B, I and J) *Dll1* (C, D, K and L) and *Hes5* (E, F, M and N) expression in the ventral hypothalamus was detected by in situ hybridization analyses of wild-type and *Ngn3*$^{-/-}$ embryos at E10.5 and E13.5. The neural tissue is outlined by dotted lines. (G, H) Immunohistochemistry analysis also showed normal Mash1 expression in *Ngn3*$^{-/-}$ embryos at E10.5, while expression of *Mash1* transcripts is not affected in *Ngn3*$^{-/-}$ embryos at E13.5 (O, P). Scale bar corresponds to 100 μm.

temperature in the second primary antibody diluted in 0.1% triton and 1% BSA in PBS, followed by washing and incubation in another secondary antibody conjugated with another fluorochrome for 2 h at room temperature. Sections were then extensively washed and mounted in DAPI-containing vectashield H-1005 (Vector Laboratories, Burlingame, CA).

The following primary antibodies were used: rabbit anti-TH (AB152, 1:100; Chemicon, Temecula, CA), sheep anti-TH (AB1542, 1:100; Chemicon, Temecula, CA), rabbit anti-SF1 (1:1000; Hatano et al., 1994, kind gift of K. Morohashi), mouse anti-ACTH (CR1096M, 1:300; Cortex Biochem Inc.), sheep anti-γMSH (1:100; kind gift of A. Bicknell), rabbit anti-GFP (1:500; Molecular Probes), sheep anti-GFP (1:400; Molecular Probes), mouse anti-Mash1 (1:200; Developmental Studies Hybridoma Bank (DSHB), IA, USA), rat anti-Ki67 (1:50, DAKO), rabbit anti-Ngn3 (1:2000, generous gift of M. German), rabbit anti-NPY (1:100, T-4070, Peninsula Laboratories, CA, USA) and mouse anti-caspase-3 active (1:1000, R&D systems). The following second-ary antibodies were used: rabbit anti-Cy3 (1:200; Jackson ImmunoR-esearch Laboratories), sheep anti-Cy3 (1:200; Jackson ImmunoResearch Laboratories), rat anti-Cy3 (1:200; Jackson Immu-noResearch Laboratories), rabbit anti-Alexa 488 (1:500; Molecular Probes), mouse anti-Alexa 594 (1:500; Molecular Probes), and mouse anti-Alexa 488 (1:500; Molecular Probes).

Image analysis and cell counting

Bright field images were obtained with a Zeiss LSM510 microscope and Axiovision 2.1 software, while confocal images were obtained with a Leica TCS SP2 confocal microscope. Images were processed using Adobe Photoshop CS (Adobe Systems, San Jose, CA).

All cell counts were carried out using the Cell Counter plug-in for ImageJ (http://rsbweb.nih.gov/ij/). Cell counts were derived from an average of three counts of cell nuclei from a single series of cryostat cut sections per animal, with a mean taken from a minimum of three series per expression pattern of interest. Except where stated, all positive cells were counted across the whole A-P axis of the expression domain of each series, and as a result cells were counted from between four and seven sections of each series. For reasons of expediency, only the left or right domain of expression was counted in each series, and the total multiplied by 2 to estimate the number of positive cells across the whole series. NPY and αMSH immunostained cells were only counted within the ARC, the boundary of which was determined by use of an anatomical atlas of the developing mouse brain and the authors' own judgment based on experience. In order to estimate if there is any change in the total number of ARC cells in the hypothalamus of *Ngn3*$^{-/-}$ mutant embryos, fluorescent immunohis-tochemical labelling for SF1 protein expression along with DAPI nuclei

staining was carried out at E17.5 and images obtained at one *z*-level for two representative sections of each series obtained. In each section Adobe Photoshop CS was used to highlight a region of interest from the most ventral boundary of SF1 expression, the boundary of the third ventricle and the ventral most boundary of the hypothalamus. The outline of this region was then exported as a new layer and applied to the image of DAPI stained cell nuclei from the same stack, and the positive staining counted, excluding those cells bisected by 50% or more by the bounding line. Co-expression with αMSH was carried out to determine whether a substantial proportion of POMC neurons would be excluded from this count at this stage of development. In all cases, *t*-tests were then carried out to assess for any significant difference in immuno-positive or dig-labelled in situ stained cells between wild-type and mutant animals.

Results

Ngn3 is expressed in a subset of progenitors and early postmitotic neurons in the ARC and VMH

Neurogenin 3 (Ngn3) transcripts are first detected in the ventricular zone of the ventral hypothalamus at E9.5 (Fig. 1A). This expression pattern expands from E10.5 onwards (Fig. 1B) with higher numbers of Ngn3+ cells in the ARC/VMH region of the hypothalamus at E12.5 (Fig. 1C) and E13.5 (Fig. 1D). Subsequently, the number of Ngn3+ cells decrease, with only a few cells expressing Ngn3 mRNA in these regions at E17.5 (Fig. 1E and F). This dynamic change in Ngn3 expression pattern during development was confirmed by analyses of the expression of Ngn3 protein by immunohistochemistry with a Ngn3-specific antibody (Fig. 1A'–F'), except that there was a delay in the onset of detectable expression of Ngn3 protein, which was not detectable at E9.5.

Since Ngn3 expression occurred in cells near or in the ventricular zone of the hypothalamus, we next determined whether Ngn3 was expressed in mitotic or postmitotic cells using double antibody labelling experiments. Some Ngn3+ cells also expressed Ki67, a marker for mitotic cells, at E10.5 and E13.5 (arrows in Fig. 2A and B, respectively). Ngn3 is also co-expressed with Mash1 (arrows in Fig. 2C, D), which labels only progenitor cells, in the hypothalamus (McNay et al., 2006). Furthermore, double-labelled Ngn3+ POMC cells were not detected indicating that Ngn3 is not expressed in postmitotic POMC ARC neurons. Altogether, these results indicate Ngn3 is expressed in progenitors in the ARC/VMH region of the hypothalamus. These results also suggest that Ngn3 is initiated slightly later and likely acts downstream of Mash1 in these progenitors. Consistent with this interpretation, Ngn3 expression is lost in *Mash1⁻/⁻* mutants at E10.5 but is recovered at E13.5 (McNay et al., 2006).

Ngn3+ progenitors contribute to subsets of ARC and VMH neuronal populations

We obtained direct evidence that Ngn3+ progenitors give rise to distinct neuronal subtypes in the ARC/VMH region by performing genetic fate mapping studies using a *Ngn3-Cre* BAC transgenic line that allowed us to permanently label any cell expressing Ngn3 and its progeny (Schonhoff et al., 2004). This Cre line faithfully mimics Ngn3 expression in the small intestine and pancreas. Double-labelling of YFP and Ngn3 by immunohistochemistry identified YFP+Ngn3+ cells in the ventricular and mantle zone (Fig. 3C), indicating that YFP is expressed in Ngn3-expressing progenitors and postmitotic neurons in the hypothalamus of *Ngn3;R26R^YFP* embryos. However, some Ngn3+, YFP- cells were also observed in more dorsal hypothalamic regions, suggesting that there is a delay in Cre and consequently YFP expression, compared to the timing of Ngn3 expression (Fig. 3A–C). This delay in the activation of reporter genes driven off Ngn3
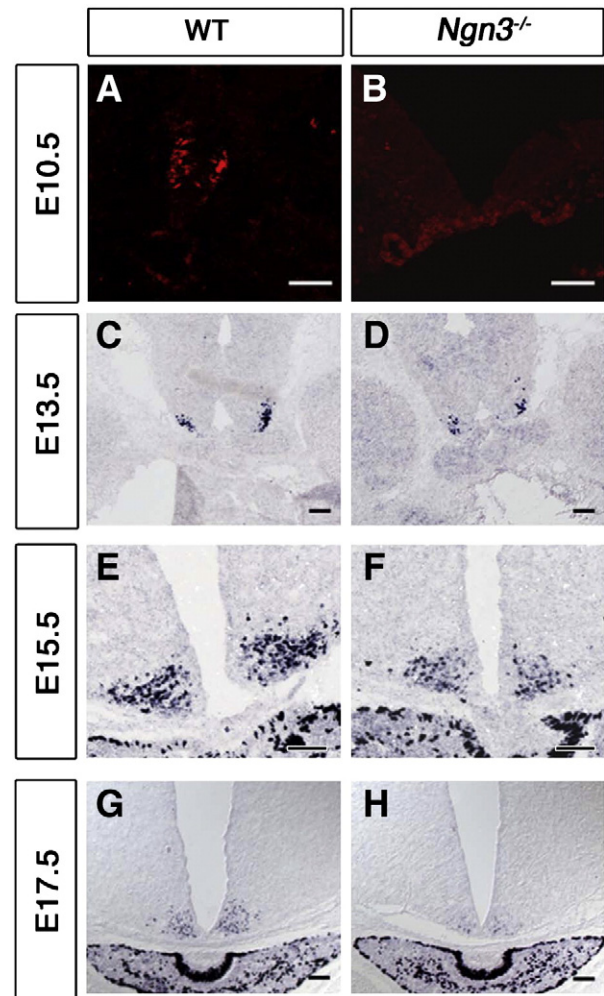

Fig. 5. Reduced numbers of POMC neurons in *Ngn3⁻/⁻* embryos throughout embryonic development. (A–H) Coronal sections of mouse embryos. Immunofluorescent staining (A, B) and in situ hybridization analyses (C–H) revealed severe reduction in the numbers of POMC neurons in *Ngn3⁻/⁻* compared to wild-type embryos from E10.5 to E17.5. (I–L). *Pomc* is also expressed in the pituitary at E15.5 and E17.5.

Table 1
Numerical data of the cell counts of ARC and VMH neuronal populations in *Ngn3⁻/⁻* mutant compared to wild-type embryos.

		Mean number of positive cells per series ($n = 3$)			
		WT		Ngn3⁻/⁻	
		Mean	SD	Mean	SD
E10.5	Pomc	103.75	6.24	20.75	14.86
	SF1	207.33	19.09	52.67	17.67
	Nhlh2	2769	357.53	302.52	198.43
E13.5	Pomc	562.39	146.74	115.28	74.32
	SF1	3695.11	545.42	2438.67	50.36
	TH	897.06	41.43	911.67	29.04
	Nhlh2	218.31	43.40	21.18	8.63
E15.5	Pomc	736.25	196.56	309.79	94.79
	Npy	351	16.52	755.67	147.41
	SF1	8551.46	733.23	5772.79	626.82
	TH	985.47	197.79	1477.47	259.29
	Nhlh2	3608	491.23	428.5	206.87
E17.5	Pomc	858.50	154.65	326.13	65.87
	Npy	747.33	58.73	1548	247.78
	SF1	10099.33	1408.77	7448.67	933.50
	TH	813.67	45.54	1189	76.31
	Nhlh2	3726	832.16	746.67	189.01

Data represent mean of total number of positive cells per series across the entire detectable expression domain of each population marker ($n = 3$ for each genotype).
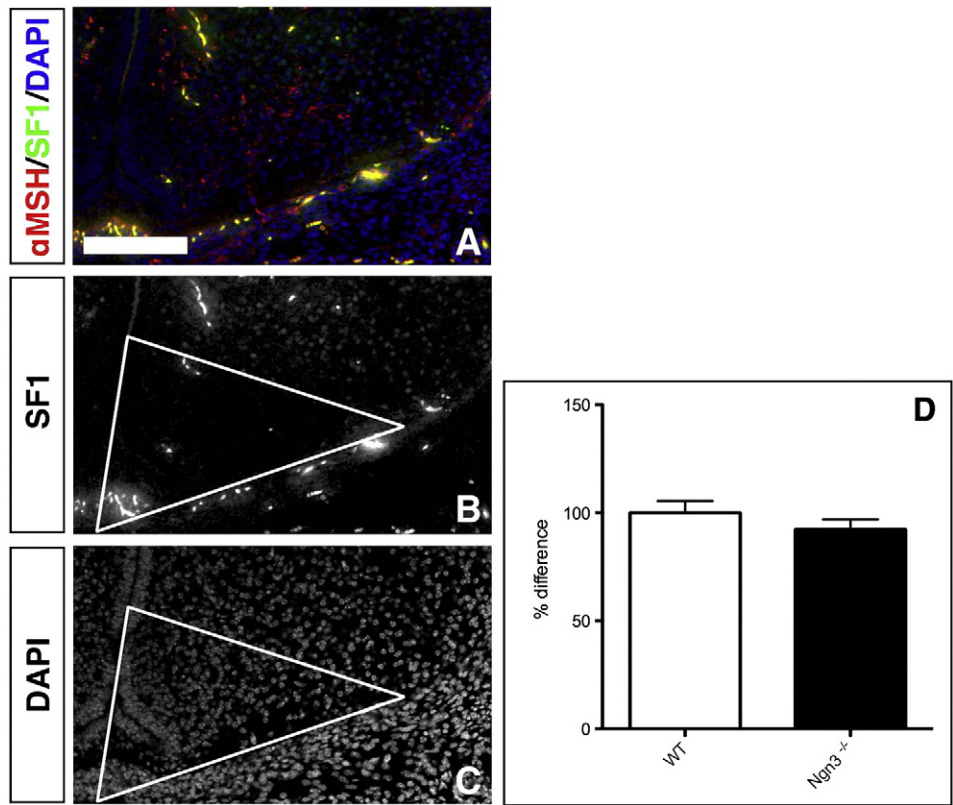
Fig. 6. Comparison of the total number of DAPI stained cell nuclei in wild-type and $Ngn3^{-/-}$ embryos at E17.5. (A) Expression of αMSH and SF1 determined by fluorescent immunohistochemical and DAPI staining. (B) Arcuate domain approximated by area ventral to SF1 expression domain boundary. (C) DAPI staining in the arcuate domain defined by SF1 expression. (D) No overall difference in total number of DAPI positive cells is observed in $Ngn3^{-/-}$ mutants compared to WT littermates ($n=3$).

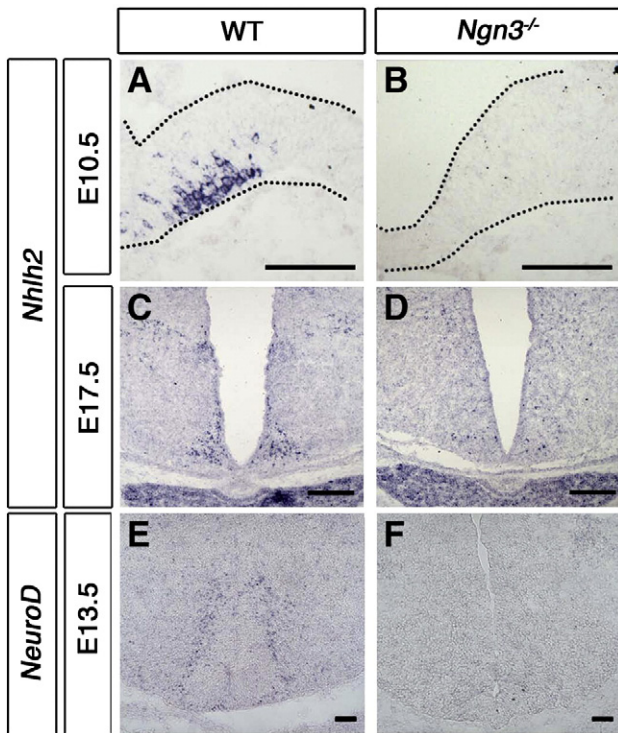Fig. 7. Two bHLH genes, *Nhlh2* and *NeuroD*, are downstream targets of Ngn3. (A–F) Coronal sections of mouse embryos. In situ hybridization of tissue sections of WT and $Ngn3^{-/-}$ mutant littermates. Expression of *Nhlh2* is severely reduced in $Ngn3^{-/-}$ mutant compared to wild-type embryos at E10.5 (A and B), and E17.5 (C and D). The neural tissue is outlined by dotted lines in A and B. *NeuroD* expression is also missing in $Ngn3^{-/-}$ mutants (F), while it is expressed in nascent neurons in the ventral hypothalamus at E13.5 (E). Scale bar corresponds to 100 μm.

promoter regulatory sequences has already been reported (Jenny et al., 2002). We first examined whether Ngn3+ progenitors generated POMC neurons in the ARC by double labelling with YFP and POMC antibodies in $Ngn3;R26R^{YFP}$ embryos. Quantitative analysis showed that the percentage of YFP+/POMC neurons over the total number of POMC neurons was 96% at E10.5 and reduced to 67% at E15.5 (Fig. 3D–F and data not shown). These results indicate that a subset of POMC neurons are derived from Ngn3+ progenitors in $Ngn3;R26R^{YFP}$ embryos and suggests that later born POMC neurons may be derived from Ngn3- progenitors.

We also investigated whether other neuronal populations in the ARC/VMH region are derived from Ngn3 progenitors. We analysed only populations where antibodies are available to perform double antibody labelling experiments with YFP. Our results demonstrate that a subset of TH dopaminergic (Fig. 3J and K), and NPY neurons in the ARC (Fig. 3L) and SF1 neurons in the ARC and VMH (Fig. 3G, H, I) are also derived from Ngn3+ progenitors. The contribution to POMC, TH and SF1 neurons at E13.5 has been confirmed using E13.5 heterozygous embryos from $Ngn3^{EYFP}$ knock-add-on (Mellitzer et al., 2004; Supplementary Fig. 1) and $Ngn3LacZ$ ((Jenny et al., 2002, data not shown) mouse lines, although the contribution to the different neuronal subtypes is lower in the hypothalamus of these embryos (data not shown) as was expected since $Ngn3^{EYP/+}$ and $Ngn3LacZ/+$ embryos mark Ngn3+ cells in a transient manner, while there is permanent labelling of Ngn3+ cells in $Ngn3Cre/+$ embryos.

Ngn3 is not required for the generation of neurons in the ARC/VMH region

Since other Ngn genes, such as Ngn1 and Ngn2, have a proneural function in the CNS, we investigated whether Ngn3 is required for neurogenesis in the ventral hypothalamus. Neuronal differentiation, determined by the expression of an overt neuronal marker SCG10,
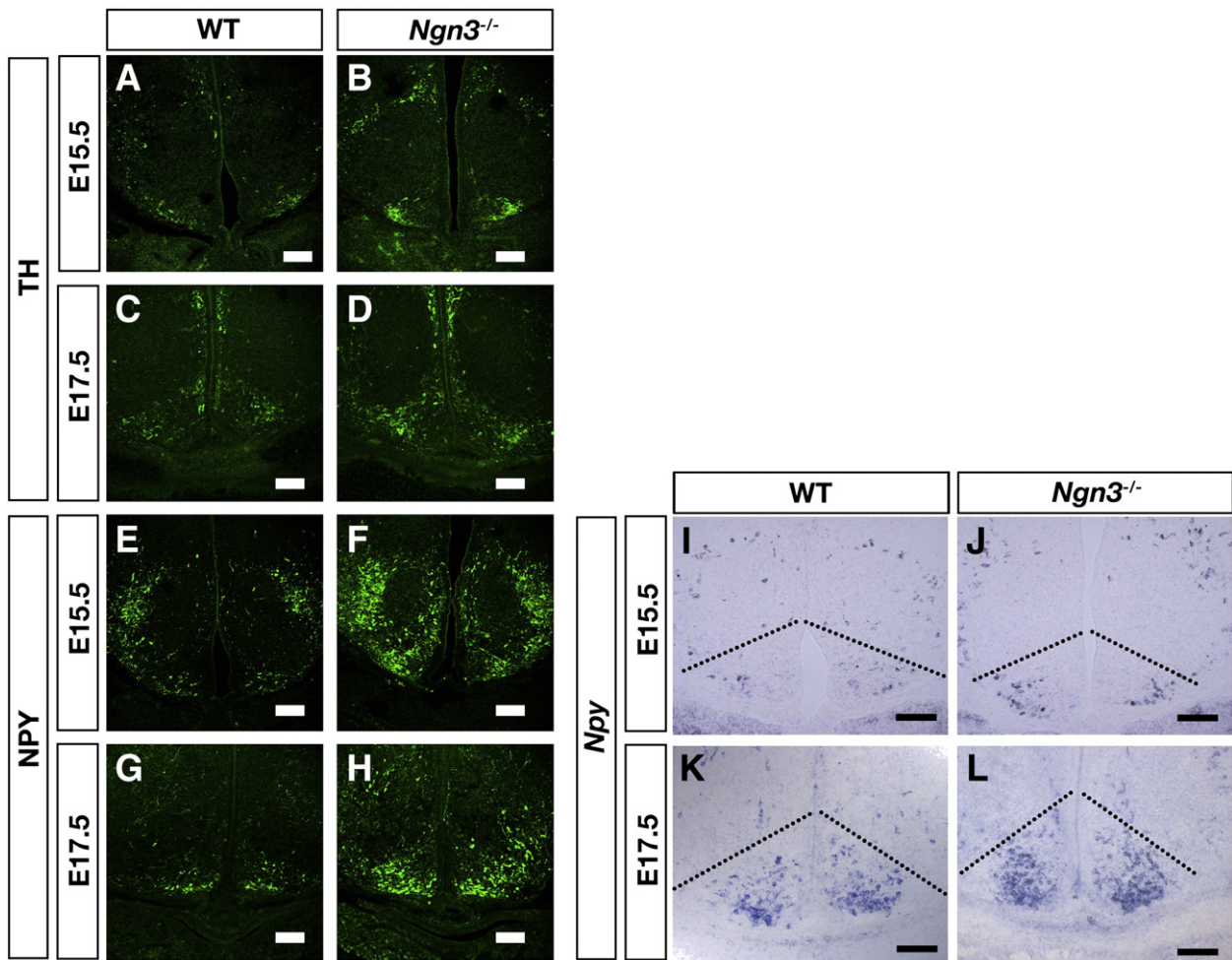
Fig. 8. Increase in the numbers of TH and NPY neurons in $Ngn3^{-/-}$ mutants from E15.5 onwards. (A–H) Coronal sections of mouse embryos. An increase in the number of TH and NPY neurons was detected by immunohistochemisty in $Ngn3^{-/-}$ mutants compared with wild-type embryos at E15.5 (A, B and E, F) and E17.5 (C, D and G, H). Similarly, an increase in the numbers of NPY neurons was observed by in situ hybridization at E15.5 (I, J) and E17.5 (K, L). Dotted lines delineate region of ARC used for the purpose of cell counting. Scale bar corresponds to 100 μm.

was unchanged in *Ngn3* mutant embryos at E10.5 and E13.5 (Fig. 4A, B, I and J). Furthermore, expression of downstream targets of proneural genes involved in lateral inhibition, such as *Dll1* and *Hes5*, were also unaffected in Ngn3 mutant compared to wild-type embryos (Fig. 4C, D, K and L). Consistent with these results, we also found that proliferation and apoptosis, measured by the expression of Ki67 and phosphorylated caspase 3, were not affected in the hypothalamus of *Ngn3* mutant embryos at both these stages (Supplementary Fig. 2). The absence of a phenotype in neurogenesis suggests that Ngn3 does not have a proneural function or it functions redundantly with Mash1, which is still normally expressed in ARC/VMH progenitors in Ngn3 mutant embryos at E10.5 and E13.5 (Fig. 4G, H, O and P), to regulate neurogenesis.

Ngn3 is required for the development of a subset of POMC neurons

POMC is one of the earliest markers of ARC neurons and is expressed from E10.5 onwards (McNay et al., 2006). Within the ARC it is processed into αMSH, which inhibits appetite, and therefore is the main anorexigenic neuropeptide of the hypothalamus. Since some POMC neurons are derived from Ngn3+ progenitors, we determined the status of these neurons by immunohistochemisty with a γMSH-specific antibody at E10.5 or by ISH to detect POMC transcripts from E13.5 onwards. Quantitative analyses revealed that 80% of POMC

neurons are lost in Ngn3 mutants compared to wild-type embryos at E10.5 and E13.5 (Figs. 5A–D and 10, Table 1). Subsequently, there is a recovery in the number of POMC neurons with only 60% of POMC neurons missing at E15.5 (Figs. 5E, F and 10, Table 1). The reduction in POMC neuronal cell number persisted at E17.5 (Figs. 5G, H and 10), which was the latest stage analyzed since *Ngn3* mutant animals die early postnatally (Gradwohl et al., 2000). However, any reduction in POMC cells at this stage was not matched by a reduction in the total number of cell bodies in the ARC (Fig. 6).

Expression of bHLH transcription factors, Nhlh2 and NeuroD are affected in the ARC

Nhlh2 (nescient helix-loop-helix 2) is a bHLH transcription factor, which is expressed in the rostral ARC POMC neurons. *Nhlh2* knockout mice develop adult-onset obesity (Coyle et al., 2002; Good et al., 1997) and Nhlh2 is required for the normal expression of the key processing enzyme, pro-hormone convertase 1 (PC1) (Jing et al., 2004). PC1 has been shown to proteolytically cleave the POMC precursor in a tissue-specific manner to produce mature POMC products, including α-Melanocyte Stimulating Hormone (α-MSH) (Nillni, 2007). In addition, Nhlh2 expression is reduced in the cortex of *Ngn2* null mutants (Schuurmans et al., 2004) and conversely forced expression of Ngn2 induced *Nhlh2* in the ventral telencephalon within
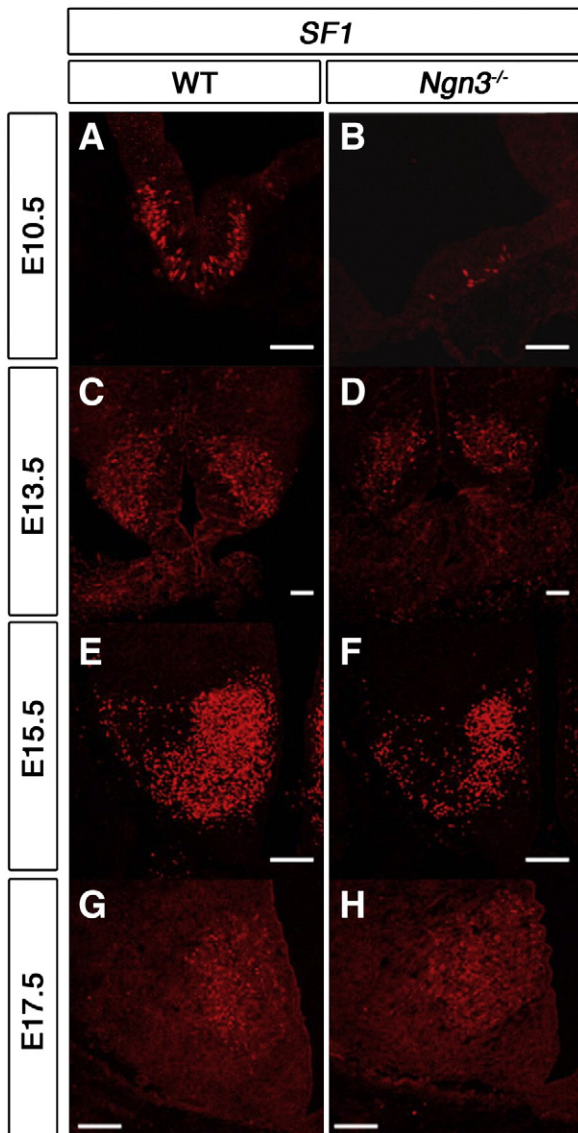
Fig. 9. SF1 neurons are also reduced in number in Ngn3$^{-/-}$ compared to wild-type embryos. (A–H) Coronal sections of mouse embryos. Immunohistochemistry analyses indicate that the number of SF1 neurons in the ARC and VMH at E10.5 (A, B) and in the VMH is reduced from E13.5 to E17.5 (C–H). Scale bar corresponds to 100 μm.

24 h (Mattar et al., 2008), indicating that *Nhlh2* is a downstream target gene of Ngn2. We therefore examined whether Ngn3 could have a similar role in regulating *Nhlh2* expression in the ventral hypothalamus. A dramatic reduction in the number of *Nhlh2* cells was observed in *Ngn3* mutants from E10.5 to E17.5 (Figs. 7A–D and 10). Quantitative analyses showed that 20% Nhlh2+ cells remained in the ARC at E17.5 (Table 1).

NeuroD, another bHLH transcription factor, has also been shown to be regulated by neurogenin genes in the nervous system (Fode et al., 2000; Ma et al., 1996) and by Ngn3 in the pancreas (Huang et al., 2000). Consistent with these results, *NeuroD* expression is also missing in Ngn3 embryos (Fig. 7E, F). Hence, the expression of several bHLH genes expressed in postmitotic neurons in the ARC appears to be dependent on Ngn3.

Expansion of TH and NPY ARC populations in the Ngn3 mutants

Since Ngn3+ progenitors also generate TH and NPY neurons in the ARC, we determined the status of these neurons in *Ngn3*$^{-/-}$ embryos. TH is the first rate-limiting enzyme for the synthesis of dopamine from tyrosine. Dopamine produced in the A12 (Ben-Jonathan and Hnasko, 2001) ARC neurons is required for providing inhibitory control of prolactin secretion. Quantitative analyses of the number of TH neurons revealed no change in the number of A12 dopaminergic neurons between WT and mutant embryos at E13.5 (Fig. 10, Table 1). However, there was a 50% increase in number of TH neurons in *Ngn3*$^{-/-}$ mutants both at E15.5 and E17.5 (Figs. 8A–D and 10, Table 1).

NPY is a neuropeptide belonging to the pancreatic polypeptide family and is one of the major orexigenic peptide expressed in the ARC. Expression of NPY causes an increase in food intake and body weight, leading to obesity. *NPY* neurons are detected from E15.5 onwards in the ARC (Fig. 8E). There is a dramatic increase in the number of *NPY* neurons in *Ngn3*$^{-/-}$ mutant embryos compared to control littermates at both E15.5 and E17.5, with a doubling in size of the population (Figs. 8E–H and 10, Table 1). Altogether, these results demonstrate that loss of Ngn3 results in changes in the ratio of neuronal subtypes in the ARC. While the numbers of POMC neurons are reduced, the numbers of NPY and TH neurons are increased in the ventral hypothalamus.

Given the role of Ngn3 in regulating endocrine cell fate in the pancreas, we also determined whether Ngn3 is also required for the development of GHRH neuroendocrine cells in the ARC. Expression of *Ghrh* appears unchanged in the ARC of *Ngn3* mutant embryos (Supplementary Fig. 3E, F). Accordingly, expressions of transcription factors *Hmx2* (Wang et al., 2004) and *Gsh2* (Mutsuga et al., 2001) which regulate the expression of GHRH in the ARC are also not affected in the absence of Ngn3 at E13.5 (Supplementary Fig. 3A–D). These results therefore indicate that Ngn3 specifically regulates the development of A12 TH neuroendocrine cells.

The SF1 population, a marker of the VMH, is also reduced in the Ngn3 mutants

SF1 is an orphan nuclear receptor that is expressed in the gonad, adrenal cortex, pituitary and brain (Ikeda et al., 1994). In the brain its expression is limited to the VMH of the hypothalamus. SF1 knockut mice lack a histologically distinct VMH and exhibit late onset obesity (Dellovade et al., 2000; Ikeda et al., 1995). SF1 specifically marks VMH at E13.5, but also labels POMC neurons in the ARC at E10.5 (McNay et al., 2006). We used SF1 as a marker to determine the status of the VMH during development. A significant reduction of SF1 neurons in the VMH was observed at E13.5, E15.5 and E17.5, and in both the ARC and VMH neurons at E10.5 in Ngn3 mutant embryos (Fig. 10, Table 1). Quantitative analysis showed that the reduction in the number of SF1 neurons was approximately 30% from E13.5 onwards (Figs. 9 and 10, Table 1). In contrast, there was no obvious difference in the numbers of Nkx2.2+ neurons in the VMH (Supplementary Fig. 4). These results indicate that Ngn3 is also required specifically for the development of SF1 but not for the expression of Nkx2.2+ neurons in the VMH.

Discussion

Cascade of bHLH genes regulate the development of POMC neurons

Ngn3 is not required for neurogenesis of the ventral hypothalamus. This conclusion is based on the fact that there are no apparent changes in cell proliferation, cell death, and neuronal cell number in the ARC and VMH in *Ngn3*$^{-/-}$ embryos during development. In addition, expression of downstream targets of proneural bHLH genes, involved in Notch-mediated lateral inhibition, such as *Dll1* and *Hes5*, was not modified in *Ngn3* mutant embryos. On the other hand, Mash1 has previously been shown to have a proneural function and to act upstream of Ngn3 to regulate neurogenesis in the ventral hypothalamus (McNay et al., 2006). Loss of Mash1 leads to a loss of *Ngn3* transcripts in ARC progenitors between E10.5 and E13.5. Contrary to this, normal expression of Mash1 was observed in the hypothalamus
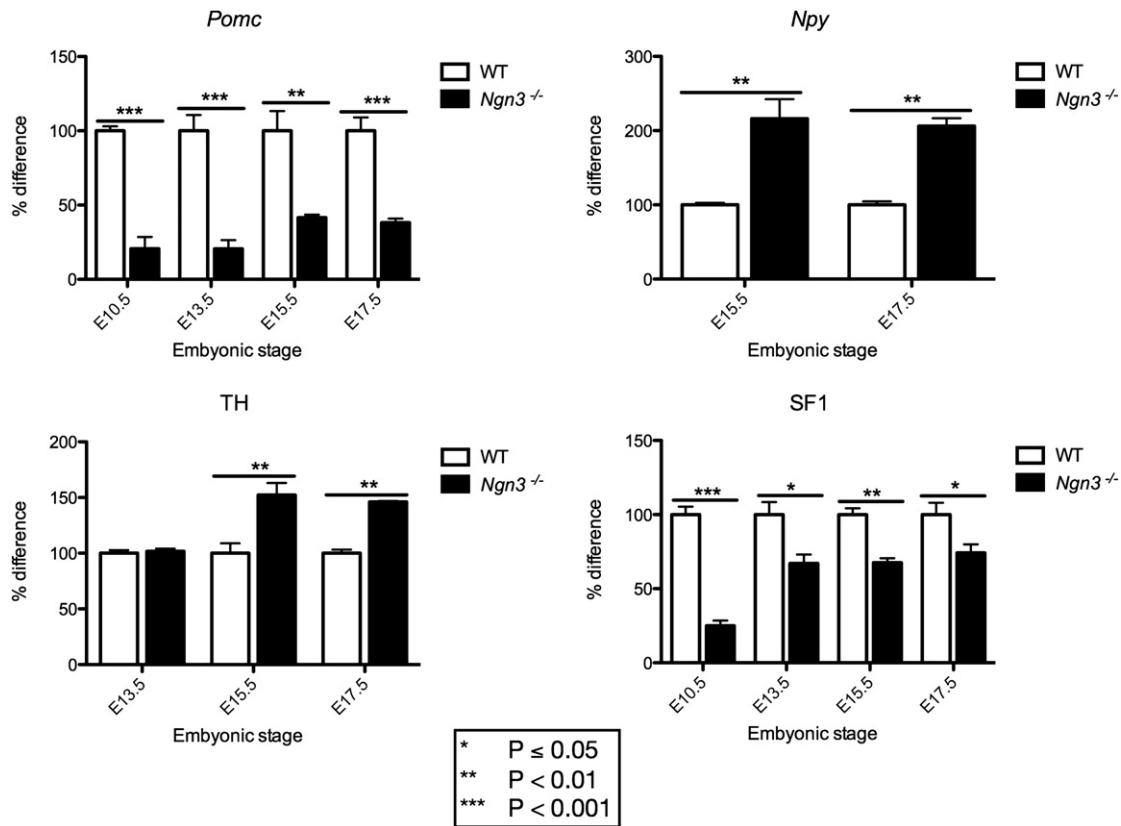
Fig. 10. Graphical representation of the changes in ARC and VMH neuronal populations in *Ngn3* mutant compared to wild-type embryos. Quantification of the number of distinct neuronal populations in $Ngn3^{-/-}$ mutants compared to wild-type embryos. There is a reduction in the percentage of POMC and SF1 neurons in $Ngn3^{-/-}$ mutants, when compared to WT embryos at all four developmental stages analyzed, although a significant recovery in the percentage of POMC neurons is seen at later developmental stages. In contrast, there is an increase in the percentage of TH and NPY neurons at E15.5 and E17.5, but not at E13.5.

of $Ngn3^{-/-}$ mutants at E10.5. Mash1 may be compensating for Ngn3 function as a proneural gene. To address this possibility, we generated double *Mash1;Ngn3* double mutants, but failed to recover any double homozygous embryos at E10.5 and E12.5 from 20 litters of $Mash1^{+/-}$; $Ngn3^{+/-}$ intercrosses. The reason for their embryonic lethality remains to be determined, but this early defect precludes the possibility of using double *Mash1;Ngn3* double mutants for determining whether Ngn3 has a proneural function during neurogenesis.

Ngn3, however, is required for the expression of two other bHLH factors *NeuroD* and *Nhlh2* that are expressed in nascent neurons outside the ventricular zone. Interestingly, NeuroD has previously been shown to be a direct target of Ngn3 in the developing pancreas and to function downstream to maintain the differentiation program initiated by Ngn3 (Huang et al., 2000). In other parts of the CNS, Ngn1 and Ngn2 also acts upstream of NeuroD to promote the differentiation of neurons in the olfactory epithelium and cerebral cortex respectively (Cau et al., 2002; Fode et al., 2000) Ngn2 also induces NeuroD and Nhlh2 expression in the ventral telencephalon (Mattar et al.,

2008). Hence, the regulatory interactions between *Neurogenins* and *NeuroD* and Neurogenins and *Nhlh2* are conserved in the hypothalamus. The function of NeuroD in the hypothalamus has not been described, while loss of Nhlh2 leads to adult-onset obesity that is in part contributed by post-translation control of the processing of the POMC propolypeptide by the PC1 convertase (Jing et al., 2004). Taken together, these studies suggest a model whereby a cascade of bHLH genes is involved in the regulation of POMC expression at the transcriptional and postranscriptional level at sequential steps in the lineage (Fig. 11).

Ngn3 is a critical determinant of subsets of ARC and VMH neurons

Our genetic fate mapping studies using *Ngn3-Cre* mice demonstrate that some but not all arcuate POMC, NPY, TH neurons and ventromedial SF1 neurons arise from Ngn3+ progenitors. These results, confirmed in fate mapping studies with two other strains of mice, Ngn3LacZ and Ngn3^{EYFP}, raise the possibility that subpopulations
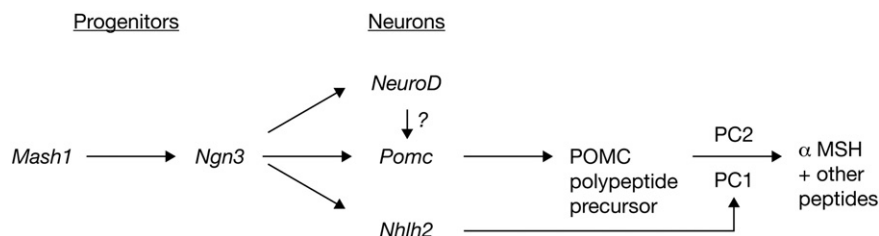

Fig. 11. Model of bHLH transcription factors regulating POMC expression at the transcriptional and postranscriptional level. *Ngn3* regulates the expression of *Pomc* and likely also αMSH peptide levels by regulating the expression of *Nhlh2*. Whether *NeuroD* is also involved in the regulation of *Pomc* remains to be determined.

of POMC, NPY, TH and SF1 neurons are generated from progenitors that do not express Ngn3 and hence are not labelled in these *Ngn3Cre; R26R*^{YFP} embryos. Alternatively, since there is a temporal delay in activation of reporter compared to the timing of Ngn3 transcription, Cre, LacZ and YFP may only label a subset of descendants from *Ngn3*$^{-/-}$ populations. Consistent with the former hypothesis, loss of Ngn3 leads to a severe but partial reduction of POMC neurons in mouse embryos during development. The numbers of POMC neurons lost in Ngn3 mutant embryos correlate well with the numbers of POMC neurons derived from Ngn3+ progenitors in the fate mapping studies at E15.5. Reduction in POMC + neuronal number is not due to a general effect on neurogenesis, which occurs normally in *Ngn3*$^{-/-}$ embryos. Consequently, the most parsimonious explanation of these results is that Ngn3 is required for the development of a subset of POMC neurons, perhaps in a cell intrinsic manner.

Remarkably, loss of Ngn3 has an opposite effect on NPY and TH neurons, leading to increase in the number of these neurons in Ngn3 mutant embryos. These results indicate that Ngn3 represses NPY and TH fate. Since Ngn3+ progenitors give rise to all three types of neurons, Ngn3 may act within a common progenitor for all three lineages, and positively select POMC fate at the expense of NPY and TH. Alternatively, distinct Ngn3+ progenitors generate unipotent POMC, NPY and TH neurons and loss of POMC neurons leads to compensatory changes in the number of other neuronal subtypes. The latter possibility is less likely given that there are no changes in the absolute number of ARC neurons at E17.5 and there is no obvious change in cell proliferation in the ARC of *Ngn3*$^{-/-}$ embryos at E10.5 and E13.5. Independent of the mechanism, our results clearly demonstrate that Ngn3 has different effects on regulating the number of POMC versus NPY and TH neurons. It is also noteworthy that while TH neuroendocrine cells are affected, there was no obvious change in the number of GHRH neuroendocrine cells. Therefore, Ngn3 is required for the development of some but not all neuroendocrine cells in the ventral hypothalamus.

Conclusion

In this paper, we have focused primarily on the role of Ngn3 in regulating the development of NPY and POMC neurons, which contribute to the regulation of food intake and energy balance in mammals. Importantly, our studies demonstrate that Ngn3 has opposite roles in regulating the development of POMC and NPY neurons. These results suggest that Ngn3 activity plays an important role in regulating the numbers of POMC and NPY neurons in the hypothalamus during development and consequently may play a role in diseases, such as obesity and Type II diabetes. Finally, it is noteworthy that besides the interplay of neuronal and hormonal mechanisms regulating energy homeostasis between the hypothalamus and the pancreas and between the hypothalamus and the gastrointestinal tract, a common molecular determinant Ngn3 governs the differentiation of endocrine cell types in all three organs.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2010.11.007.

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