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

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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.

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 (Kronenberg 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

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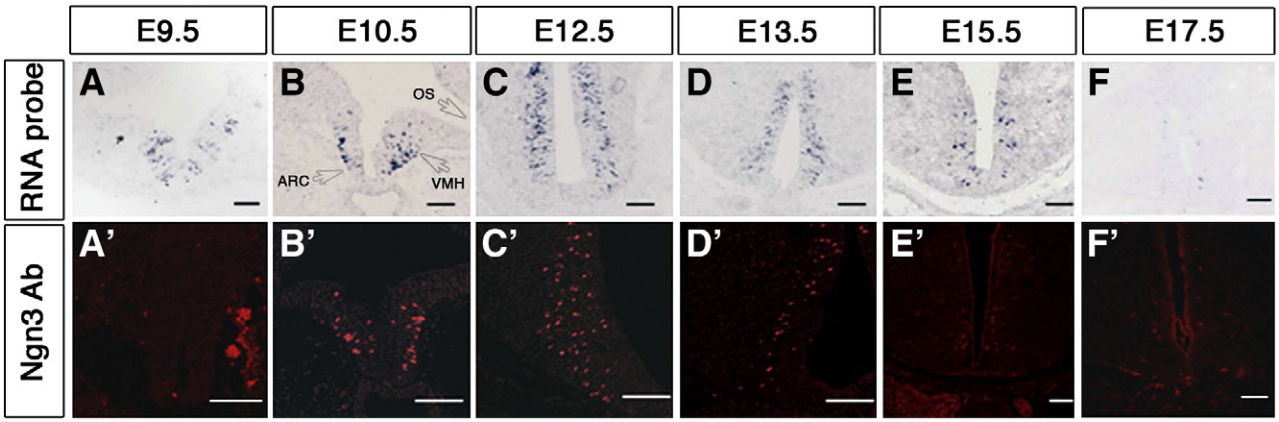

Fig. 1. Ngn3 mRNA and protein expression pattern in the ventral hypothalamus. (A–F') Coronal sections of mouse embryos. Ngn3 transcripts and protein are similarly expressed in the lateral margins of the ventricular zone surrounding the third ventricle of the VMH and ARC in the hypothalamus from E9.5 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).

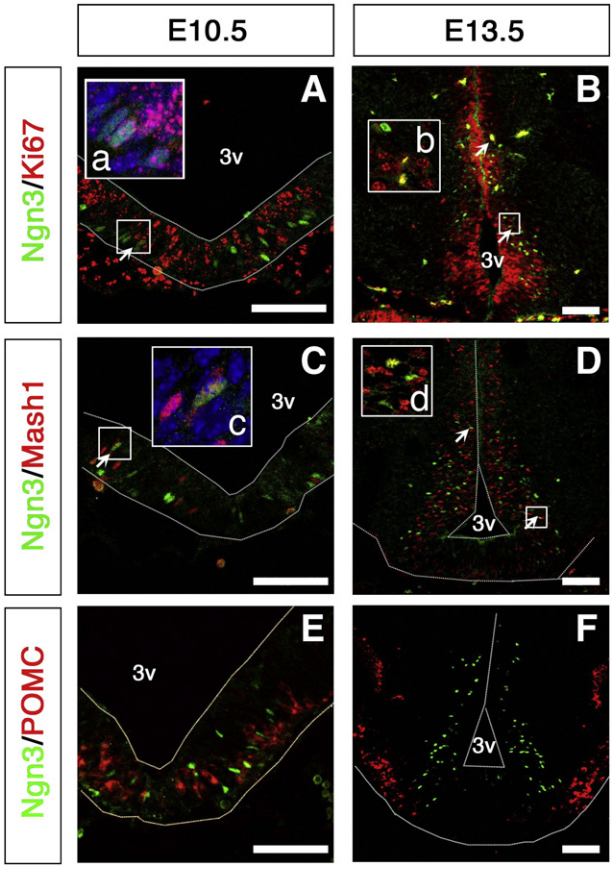

Fig. 2. Ngn3 is expressed in ventral hypothalamic progenitors. (A–F) Coronal sections of mouse embryos. (Inserts a–d) Higher magnification of the boxed region in A–D with nuclei counterstained with DAPI (blue). (A–D) Double immunofluorescent staining reveals that Ngn3 is co-expressed with mitotic markers, 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.