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Notch/Rbpjκ signaling regulates progenitor maintenance and differentiation of hypothalamic arcuate neurons

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SUMMARY

The hypothalamic arcuate nucleus (Arc), containing pro-opoiomelanocortin (POMC), neuropeptide Y (NPY) and growth hormone releasing hormone (GHRH) neurons, regulates feeding, energy balance and body size. Dysregulation of this homeostatic mediator underlies diseases ranging from growth failure to obesity. Despite considerable investigation regarding the function of Arc neurons, mechanisms governing their development remain unclear. Notch signaling factors such as *Hes1* and *Mash1* are present in hypothalamic progenitors that give rise to Arc neurons. However, how Notch signaling controls these progenitor populations is unknown. To elucidate the role of Notch signaling in Arc development, we analyzed conditional loss-of-function mice lacking a necessary Notch co-factor, Rbpjκ, in *Nkx2.1*-cre-expressing cells (*Rbpjκ* cKO), as well as mice with expression of the constitutively active *Notch1* intracellular domain (NICD) in *Nkx2.1*-cre-expressing cells (NICD Tg). We found that loss of *Rbpjκ* results in absence of *Hes1* but not of *Hes5* within the primordial Arc at E13.5. Additionally, *Mash1* expression is increased, coincident with increased proliferation and accumulation of Arc neurons at E13.5. At E18.5, *Rbpjκ* cKO mice have few progenitors and show increased numbers of differentiated Pomc, NPY and Ghrh neurons. By contrast, NICD Tg mice have increased hypothalamic progenitors, show an absence of differentiated Arc neurons and aberrant glial differentiation at E18.5. Subsequently, both *Rbpjκ* cKO and NICD Tg mice have changes in growth and body size during postnatal development. Taken together, our results demonstrate that Notch/Rbpjκ signaling regulates the generation and differentiation of Arc neurons, which contribute to homeostatic regulation of body size.

KEY WORDS: Arcuate, Notch, POMC, NPY, Hypothalamus, Mouse

INTRODUCTION

The mammalian hypothalamus regulates essential and dynamic physiological functions, including growth, metabolism, sleep, as well as reproductive behaviors (Kronenberg et al., 2007; Caqueret et al., 2005; Michaud, 2001). The hypothalamus comprises of complex networked sets of nuclei that regulate homeostatic function through production and delivery of neuropeptide to distinct targets (Swanson and Sawchenko, 1983). A specific group of hypothalamic neurons, which are located in the arcuate nucleus (Arc), integrate peripheral signals and project to other brain regions in order to regulate energy balance and body size (Akimoto et al., 2010).

Developmental defects in the Arc and related hypothalamic nuclei within the ventral hypothalamus may contribute to multiple diseases, including obesity and related metabolic disorders (Caqueret et al., 2005; Krude et al., 1998; O'Rahilly, 2009). Previous studies have identified morphogens and genes responsible for the patterning of cells along the developing dorsoventral and anterioposterior axes (Alvarez-Bolado et al., 2012; Blackshaw et al., 2010; Dale et al., 1997; Lee et al., 2006; Morales-Delgado et al., 2011; Ohyama et al., 2008; Shimogori et al., 2010). Additional studies have identified genes necessary for specification of neuronal subtypes within the ventral hypothalamus (Acampora et al., 1999; Li et al., 1996; Marin et al., 2000; McNay et al., 2006; Pelling et al., 2011). However, signals that direct expression of these factors or directly control hypothalamic neurogenesis have not been identified.

Hypothalamic neurogenesis begins on embryonic day 10.5 (E10.5) in the mouse, peaking at E12.5 (Ishii and Bouret, 2012; Shimada and Nakamura, 1973). Cells that form the Arc are born along the anterior region of the ventral surface, lining the third ventricle (Shimogori et al., 2010). Recent studies have shown that Notch signaling pathway factors are found in Arc progenitors during embryonic development *in vivo* (Kita et al., 2007; Pelling et al., 2011; Shimogori et al., 2010), as well as in proliferating fetal hypothalamic neural stem/progenitor cells *in vitro* (Desai et al., 2011a; Desai et al., 2011b).

Activation of Notch signaling occurs when the Notch intracellular domain (NICD) is cleaved and translocates to the nucleus and associates with the Rbpjκ mastermind (MAM) complex (Selkoe and Kopan, 2003). Within the nucleus, the NICD/Rbpjκ/MAM induces transcription of basic helix-loop-helix (bHLH) transcription factors encoded by Hes and Hey genes (Iso et al., 2003). Adding complexity to the system, Notch can signal independently of Rbpjκ/MAM, and Hes genes induced by signals other than Notch/Rbpjκ and Rbpjκ/MAM can also have transcriptional activity independently of Notch (Brennan and Gardner, 2002; Ingram et al., 2008; Johnson and Macdonald, 2011; Martinez Arias et al., 2002; Wall et al., 2009).

Notch targets *Hes1* and *Hes5* are essential in regulating progenitor pool maintenance during development of the neocortex (Hitoshi et al., 2002; Yoon et al., 2004; Yoon and Gaiano, 2005). Loss of *Hes1* and *Hes5* result in progenitor depletion and promote premature neuronal differentiation (Kageyama et al., 2005; Yoon and Gaiano, 2005). By contrast, increased or persistent activation of *Notch1* promotes maintenance of a progenitor state and inhibits neuronal differentiation (Gaiano et al., 2000; Mizutani and Saito, 2005). Taken together with reports of *Hes1* and *Hes5* in the developing Arc (Kita et al., 2007; McNay et al., 2006; Pelling et al., 2011; Shimogori et al., 2010), we hypothesize that Notch/Rbpjκ signaling may play a role in maintaining progenitors and controlling differentiation of Arc neurons.

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During embryonic development, cells migrate from the hypothalamic ventricular zone (HVZ) surrounding the ventral region of the third ventricle in order to form the Arc by E16.5 (Bayer and Altman, 1987; Ishii and Bouret, 2012; Shimada and Nakamura, 1973). One of the major functions of the Arc is to respond to food intake and energy expenditure. Energy-related hormone signals such as leptin are sensed by anorexic pro-opoiomelanocortin (POMC)/cocaine and amphetamine-regulated transcript (CART) neurons and orexic neuropeptide Y (NPY)/Agouti-related peptide (AgRP) neurons. POMC and NPY neurons regulate feeding behavior and are crucial to maintaining proper energy balance and homeostasis (Broberger, 2005; Morton et al., 2006; Srinivas et al., 2001). An additional subtype of neurons, growth hormone-releasing hormone (GHRH) neurons, are present in the Arc and regulate body size and growth by controlling release of growth hormone from the pituitary gland (Bouyer et al., 2007; Grossman et al., 1986).

Despite the functional importance of the Arc, little is known about the factors that control Arc neuron differentiation during development. To explore whether Notch/Rbpjκ signaling is involved in the development of the Arc neurons, we used a loss- and gain-of-function approach. We analyzed mice with loss of Rbpjκ (*Rbpj* – Mouse Genome Informatics; *Rbpj*κ cKO), as well as mice with persistent expression of NICD (NICD Tg) specifically within *Nkx2.1*-positive cells. Neurons and glia that form the Arc originate from the *Nkx2.1*-positive region of the proliferating third ventricle (Shimada and Nakamura, 1973; Shimogori et al., 2010), and fate-mapping studies have shown that *Nkx2.1* cells and their lineages are expressed within Arc neurons (Yee et al., 2009). Given the timing and spatial restriction of *Nkx2.1* expression (Lazzaro et al., 1991; Nakamura et al., 2001; Ring and Zeltser, 2010), these mice provide a useful model for examining Arc Notch/Rbpjκ pathway loss, as well as constitutive Notch activation.

We found that loss of Rbpjκ results in absence of *Hes1* expression and increased *Mash1* (*Ascl1* – Mouse Genome Informatics) expression, corresponding with increased proliferation and differentiation of Arc neurons at E13.5. At E18.5, *Rbpj*κ cKO mice have few progenitors and increased numbers of differentiated Arc neurons. By contrast, NICD Tg mice have an expanded hypothalamic progenitor population and absence of differentiated Arc neurons at E18.5. Our results suggest that Notch/Rbpjκ signaling regulates the generation and differentiation of Arc neurons.

MATERIALS AND METHODS

Mice

$Rosa^{NotchICD}$ floxed mice (Murtaugh et al., 2003) purchased from Jackson Laboratories (Bar Harbor, ME, USA) and *Rbpj*κ floxed mice (Dr Tasuku Honjo, Kyoto University, Japan) (Han et al., 2002) were bred to *Nkx2.1*-cre mice (Lazzaro et al., 1991; Xu et al., 2008) purchased from Jackson Laboratories. Breeding colonies were generated at the University of Illinois at Urbana-Champaign (UIUC) and all animal procedures were approved by the UIUC Institutional Animal Care and Use Committee. Genotyping was performed as described previously (Han et al., 2002; Lazzaro et al., 1991; Murtaugh et al., 2003).

Histology, immunohistochemistry and *in situ* hybridization

$Rosa^{NotchICD/+}$ mice (NICD Tg Control), $Rosa^{NotchICD/+}$ *Nkx2.1*-$cre^{+/cre}$ (NICD Tg), *Rbpj*$\kappa^{fl/fl}$ *Nkx2.1*-$cre^{+/+}$ (*Rbpj*κ cKO Control), *Rbpj*$\kappa^{fl/fl}$ *Nkx2.1*-$cre^{+/cre}$ (*Rbpj*κ cKO) mice were collected at E13.5, E18.5 and day of parturition (P1) and fixed in 3.8% formaldehyde (Fisher, Pittsburg, PA, USA) in phosphate-buffered saline (PBS). All experiments included littermate controls for each genotype (n=3-5). Samples were dehydrated, embedded, sectioned and deparaffinized as previously described (Goldberg et al., 2011). Hematoxylin and Eosin stain was used to observe cell morphology. For antigen retrieval, slides incubated with anti-Sox2, anti-Ki67, anti-nestin, anti-BrdU, anti-NPY, anti-GFAP and anti-TH antibody were placed in boiling 10 mM citric acid (pH 6) for 10 minutes. Slides were then incubated in normal donkey serum [5% (wt/vol)] diluted in immunohistochemistry block, consisting of PBS, BSA (3%), and Triton X-100 (0.5%), followed by overnight incubation at 4°C with a primary antibody against the desired peptide: mouse anti-BrdU (BD Pharmingen, San Jose, CA, USA; 1:150), rat anti-Ki67 (DAKO, Carpinteria, CA, USA; 1:100), rabbit anti-POMC (DAKO, Carpinteria, CA, USA; 1:1000), rabbit anti-Sox2 antibody (Millipore, Billerica, MA, USA; 1:500), rabbit anti-GFAP (Neomarkers, Fremont, CA, USA; 1:500), rabbit anti-NPY (Peninsula Labs, San Carlos, CA, USA; 1:10,000), rabbit anti-TH (Millipore, Billerica, MA, USA; 1:1000) and mouse anti-nestin (Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA; 1:100). Donkey-derived mouse, rat and rabbit secondary antibodies conjugated to biotin (Jackson ImmunoResearch, West Grove, PA, USA) were diluted to 1:200 and incubated with sections for 1 hour. Slides were then incubated for 1 hour with tertiary antibodies that were streptavidin conjugated to cy2 or cy3 fluorophore (Jackson ImmunoResearch). All sides were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma, St Louis, MO, USA) at 1:1000 (1 mg/ml) and mounted using fluorescence mounting media.

Cell death was determined by the TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) method using the *in situ* cell death detection kit (Roche, Indianapolis IN, USA) according to the manufacturer's protocol.

For *in situ* hybridization, embryos were collected, embedded and deparaffinized as they were for immunohistochemistry. The *in situ* probes used were against *Hes1*, *Hes5*, *Notch2* (Akazawa et al., 1992), *Mash1* (Carninci et al., 2003), *Notch1* (a gift from Dr Andy Groves, Baylor College of Medicine, Houston, TX, USA), *Nkx2.1* (a gift from Dr Lori Sussel, Columbia University, New York, NY, USA), *Rax* (a gift from Dr Seth Blackshaw, Johns Hopkins University, Baltimore, MD, USA) (Shimogori et al., 2010), *Pomc* (a gift from Dr Malcom Low, University of Michigan, Ann Arbor, MI, USA) (Japón et al., 1994), *Ghrh* (a gift from Dr Paul Le Tissier, MRC NIMR, London, UK) (Balthasar et al., 2003; Le Tissier et al., 2005). Probes were linearized and transcribed with polymerase in the presence of digoxigenin-labeled nucleotides. Slides were acetylated, incubated in 2×hybridization solution (Sigma) and deionized formamide, and incubated at their respective hybridization temperature for several hours. The probes were denatured for 3 minutes and applied on slides overnight at hybridization temperature. Slides were then put in a 50% formamide 0.5× sodium citrate solution at hybridization temperature for 1 hour. The slides were then blocked with 10% heat-inactivated sheep serum in Tris-buffered saline containing 2% BSA and 0.1% Triton X-100, followed by application of antidigoxigenin antibody conjugated to alkaline phosphatase (Roche, Indianapolis, IN, USA). NBT-BCIP (Roche, Indianapolis, IN, USA; 1:50) was added overnight for detection. Samples were visualized at ×200 magnification using a DM 2560 microscope (Leica, Wetzlar, Germany) and images were obtained using Q Capture Pro software (QImaging, Surrey, British Columbia, Canada) and processed using Photoshop software (Adobe, San Jose, CA, USA).

POMC and Ki67 cell counts

POMC-immunopositive and Ki67-immunopositive cell counts were performed on 50 μm mid-sagittal sections within the total Arc (spanning ~200 μm) in NICD Tg control, NICD Tg, *Rbpj*κ cKO control and *Rbpj*κ cKO mice (n=3) at E13.5. For each animal, a total of three slides, two sections per slide, were chosen 12 μm apart. The number of Arc POMC-positive and Ki67-immunopositive cells were counted in each section and the mean and standard deviation of these averages was obtained for all groups.

Pomc and NPY cell counts

Pomc-positive and NPY-immunopositive cell counts were performed on coronal sections throughout the rostrocaudal extent of the Arc at in NICD Tg control, NICD Tg, *Rbpj*κ cKO control, and *Rbpj*κ cKO mice (n=4) at

E18.5. For each animal, a total of six slides, three sections per slide, were chosen ~50 μm apart spanning the presumptive Arc. The number of Arc *Pomc*-positive and NPY-immunopositive cells were counted in each section and the mean and standard deviation of these averages was obtained for all groups. For all cell counts, two-tailed *t*-tests were performed in Microsoft Excel to determine statistical significance.

Measurement of postnatal body weight

NICD Tg control, NICD Tg, *Rbpj*κ cKO control and *Rbpj*κ cKO (n=8) male and female mice were weighed weekly from the first week of age to 8 weeks. Mice were weaned at 3 weeks and given *ad libitum* access to normal chow (5% fat). Means for sex-matched individuals from each genotypic group were obtained and paired *t*-tests were used with alpha level of 0.05 to determine difference between the weights of control and transgenic mice in each group (Microsoft Excel 2011). Representative photographs were taken of female mice at 2 weeks and 8 weeks.

RESULTS

Notch receptors and ligands are present within the developing Arc

In order to determine the localization of Notch receptors and ligands in the developing Arc, we performed *in situ* hybridization probing for the Notch receptors *Notch1* and *Notch2*, as well as Notch targets *Hes1* and *Hes5* throughout the ventral hypothalamus at E13.5. Sagittal sections of the ventral hypothalamic midline contain the anterior ventral (AV) and posterior ventral (PV) hypothalamus (Fig. 1A). *Nkx2.1*, a putative marker of the ventral hypothalamus, is expressed throughout the AV and PV (Fig. 1B). *Pomc* expression within the AV hypothalamus delineates the Arc region within the AV (Fig. 1C). Both *Notch1* and *Notch2* transcripts are localized to hypothalamic ventricular zone (HVZ) of the Arc (Fig. 1D,E). The Notch target *Hes1* is found within both the AV and PV (Fig. 1F), whereas *Hes5* mRNA is localized to the Arc HVZ and not found in the PV (Fig. 1G). We also examined expression of an additional Notch target, *Hey1*, and found that it was not expressed within the ventral hypothalamus at E13.5 (data not shown). Our results indicate that Notch receptors, as well as the targets *Hes1* and *Hes5*, are localized to the HVZ of the developing Arc at E13.5 and may contribute to development of the nucleus.

Loss of *Rbpj*κ and persistent expression of *Notch1* affects *Hes1* expression and disrupts Arc morphology

In order to determine how Notch signaling affects hypothalamic progenitors within the Arc, we generated a mouse model to manipulate Notch/Rbpjκ signaling in *Nkx2.1*-positive cells. We found that *Nkx2.1* expression overlaps with expression of Notch signaling receptors and targets (Fig. 1) and *Nkx2.1*-positive cells and their lineages are broadly expressed in neuronal subtypes within the Arc (Yee et al., 2009). We then examined mice with loss of the *Notch1* receptor (Yang et al., 2004) in *Nkx2.1* cells and found that Arc progenitors were not affected (data not shown), indicating that loss of *Notch1* alone does not affect Arc formation. We therefore crossed *Rbpj*κ floxed mice (Han et al., 2002) with *Nkx2.1*-cre mice (Lazzaro et al., 1991) (*Rbpj*κ cKO) to determine how loss of the necessary Notch co-factor *Rbpj*κ would affect Arc progenitors. Additionally, we crossed $Rosa^{NotchICD/+}$ mice (Murtaugh et al., 2003) with *Nkx2.1-cre* mice to generate gain of function transgenic (NICD Tg) mice and examine how persistent expression of the active *Notch1* intracellular domain would affect Arc development.

We found that *Rbpj*κ cKO mice have an expanded Arc region with a variably sized ventricular zone (Fig. 2B) and NICD Tg mice have a reduced Arc region (Fig. 2C) compared with controls

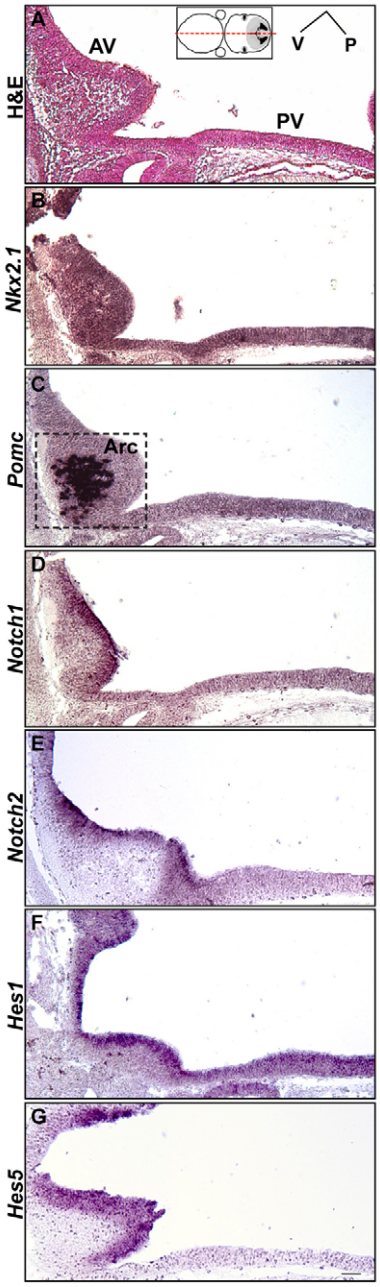

Fig. 1. Localization of Notch receptors and target genes in the developing ventral hypothalamus at E13.5. (**A**) Hematoxylin and Eosin staining on sagittal sections through the ventral midline at embryonic day 13.5 (E13.5) include the anterior ventral (AV) and posterior ventral (PV) hypothalamus. (**B**) *In situ* hybridization shows that *Nkx2.1* mRNA is expressed in hypothalamic cells within the anterior and posterior ventral midline. (**C-G**) *Pomc* mRNA expression is detectable within the primordial arcuate nucleus (Arc; C). Additionally, *Notch1* (D) and *Notch2* (E) mRNAs are expressed in the AV hypothalamus, as well as Notch targets *Hes1* (F) and *Hes5* (G). Scale bar: 50 μm.

(Fig. 2A). *Notch1* is present within the Arc HVZ of control (Fig. 2D), *Rbpj*κ cKO mice (Fig. 2E) and increased in NICD Tg mice (Fig. 2F). *Notch2* mRNA is also expressed within the Arc HVZ of control (Fig. 2G), *Rbpj*κ cKO (Fig. 2H) and NICD Tg (Fig. 2I) mice. The Notch target *Hes1* is expressed within the control Arc (Fig. 2J), absent from *Rbpj*κ cKO mice (Fig. 2K) and is present

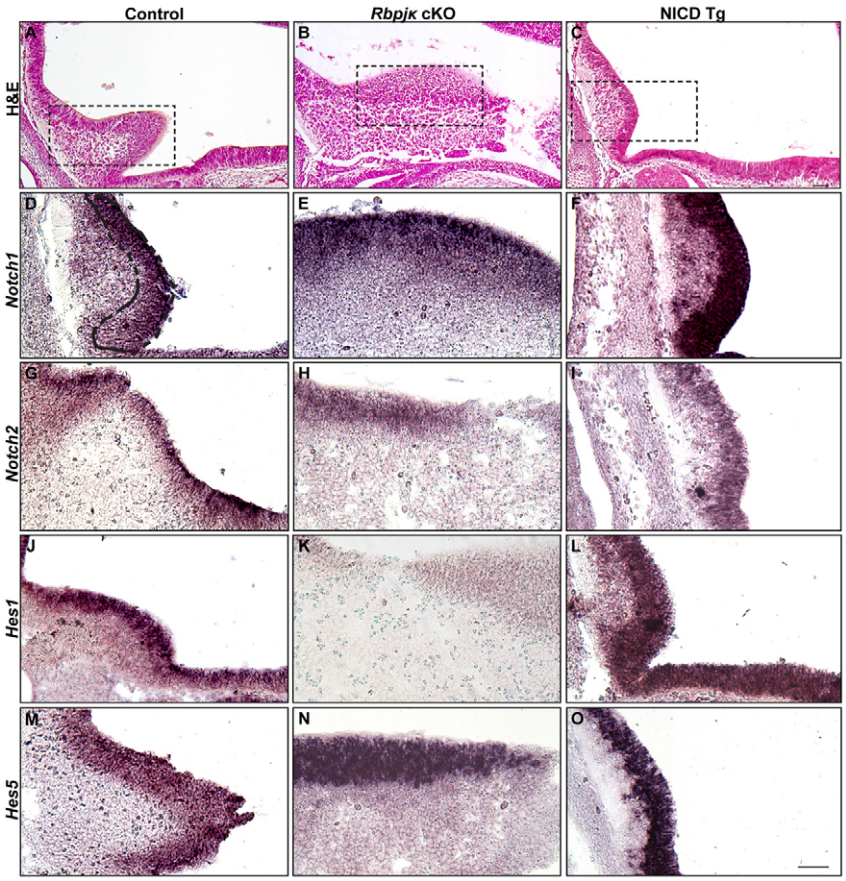

Fig. 2. Loss of Rbpjκ as well as persistent expression of *Notch1* within the developing Arc affects *Hes1* expression and disrupts Arc morphology at E13.5. (**A**) Hematoxylin and Eosin staining on sagittal sections at the ventral midline include the primordial Arc (dashed box). (**B**,**C**) *Rbpjκ* cKO mice display an expansion (B) of the primordial Arc region (dashed boxes), whereas NICD Tg mice show a reduction (C) in Arc size. (**D**) *In situ* hybridization shows that *Notch1* is expressed in the ventricular zone of the Arc (outlined). (**E**,**F**) *Notch1* expression is maintained in this region in *Rbpjκ* cKO mice (E) and is abundantly expressed within the Arc progenitor region of NICD Tg mice (F). (**G-I**) *Notch2* is also expressed in the within the Arc of control (G), *Rbpjκ* cKO (H) and NICD Tg (I) mice. (**J-L**) *Hes1* is expressed within the control Arc (J), absent from *Rbpjκ* cKO mice (K) and expressed within an expanded region within the Arc of NICD Tg mice (L). (**M-O**) Surprisingly, *Hes5* mRNA is expressed in the same region within the Arc of control animals (M), *Rbpjκ* cKO mice (N) and NICD Tg mice (O). Scale bar: 50 μm.

within the Arc of NICD Tg mice (Fig. 2L). *Hes5* mRNA is expressed along the Arc HVZ of control animals (Fig. 2M), remains expressed in *Rbpjκ* cKO mice (Fig. 2N) and is also expressed in NICD Tg mice (Fig. 2O). Taken together, our results indicate that loss of Rbpjκ leads to an expansion of the Arc, accompanied by an absence of *Hes1*, without loss of *Hes5,* suggesting that *Hes5* expression may be independent of Notch/Rbpjκ signaling in the developing Arc.

Notch signaling affects Arc progenitor population maintenance and *Pomc* differentiation

To investigate the function of Notch/Rbpjκ signaling in Arc progenitors, we probed for markers of progenitor cells, proliferation, cell death and differentiation. H&E staining shows expansion of the Arc region found in *Rbpjκ* cKO mice (Fig. 3B) and reduction of the Arc region found in NICD Tg mice (Fig. 3C) compared with controls (Fig. 3A). Importantly, the Arc HVZ is maintained within all genotypes at this age (dashed lines). The ventricular zone is populated by progenitors (Altman and Bayer, 1978; Bayer and Altman, 1987; Shimada and Nakamura, 1973), therefore, we examined Sox2 expression, a transcription factor essential for inhibiting cell differentiation to maintain pluripotency and a known marker of neural progenitor cells (Episkopou, 2005; Masui et al., 2007). We found that Sox2 is present within the ventricular zone and Arc of control (Fig. 3D), *Rbpjκ* cKO (Fig. 3E) and NICD Tg (Fig. 3F) mice at E13.5.

Proliferation of progenitors is an important aspect of progenitor maintenance and we therefore examined Ki67-immunopositive cells lining the third ventricle to detect actively dividing cells. We found that few cells in the Arc are Ki67 immunopositive in controls (21.3±2.1; Fig. 3G). However, Ki67-immunopositive cells, present within the ventricular zone (dashed lines) and diffusely expressed throughout the increased Arc of *Rbpjκ* cKO mice, are significantly increased in number (298.7±14.4, $P<0.001$; Fig. 3H). We also found that persistent expression of Notch results in significantly increased Ki67-immunopositive cells, which are localized to the dorsal aspect of the Arc (80.9±7.1, $P<0.001$; Fig. 3I).

To investigate the composition of the Arc in both *Rbpjκ* cKO and NICD Tg mice, we probed for expression of *Pomc*, the first neuron type to differentiate in the presumptive Arc region (McNay et al., 2006; Padilla et al., 2010; Pelling et al., 2011). *Pomc* mRNA is expressed within the Arc of control mice at E13.5 (Fig. 3J), diffusely expressed within the expanded Arc of *Rbpjκ* cKO mice (Fig. 3K) and expressed in few cells within NICD Tg mice (Fig. 3L). Counting of Pomc-immunopositive cells reveals that Pomc-immunopositive cells are significantly increased in *Rbpjκ* cKO mice (198.8±5.3; $P<0.001$) and significantly decreased in NICD Tg mice (10.2±0.8; $P<0.001$) compared with controls (127.8±5.2; images not shown).

Given the increase in cell proliferation and differentiation observed in the Arc of *Rbpjκ* cKO mice, we investigated the expression of *Mash1*, which is repressed by *Hes1* (Sasai et al., 1992) and is known to promote both progenitor proliferation (Castro et al., 2011) and neuronal commitment (Casarosa et al., 1999). We found that *Mash1* is increased in *Rbpjκ* cKO mice (Fig. 3N) and reduced within the ventral aspect of the Arc in NICD Tg mice (Fig. 3O) compared with controls (Fig. 3M). These data suggest that in the absence of Notch/Rbpjκ, progenitor proliferation is increased and there is robust differentiation into *Pomc*-expressing neurons, whereas persistent *Notch1* expression suppresses differentiation.

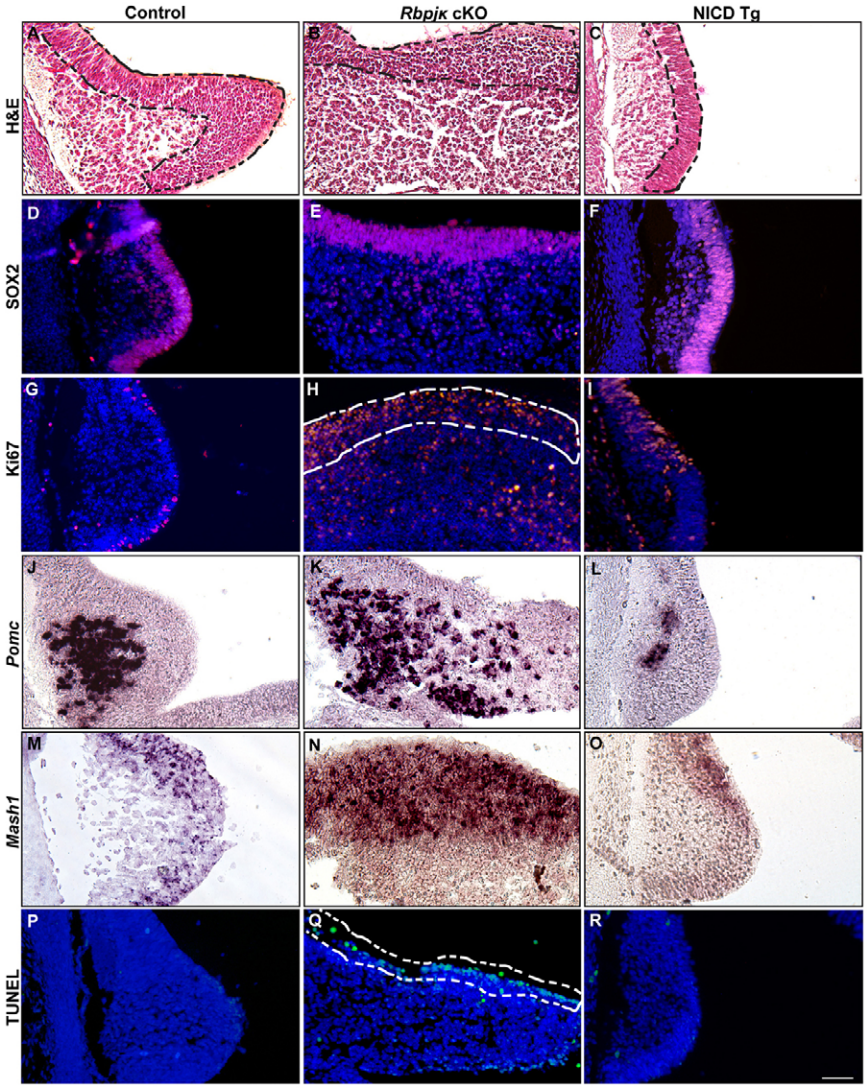

Fig. 3. Notch signaling affects Arc progenitor population maintenance and *Pomc* differentiation at E13.5. (**A-C**) Hematoxylin and Eosin staining on Arc sagittal sections shows the Arc of control (A), *Rbpj*κ cKO (B) and NICD Tg (C) mice; dashed lines outline the more densely packed ventricular zone. (**D-F**) Immunohistochemistry probing expression of the progenitor marker Sox2 shows that cells within the ventricular zone are Sox2 positive within control (D), *Rbpj*κ cKO (E) and NICD Tg (F) mice. (**G**) Some cells within the Arc HVZ are Ki67 immunopositive in control mice. (**H**,**I**) Ki67-immunopositive cells are significantly increased in number in the Arc of *Rbpj*κ cKO mice ($P<0.001$; H) and within the NICD Tg Arc ($P<0.001$), but are restricted to a dorsal position (I). (**J-L**) Arc *Pomc* mRNA is detected in control mice (J). *Rbpj*κ cKO mice have a diffuse expression of *Pomc*-positive cells throughout the expanded Arc region (K), whereas NICD Tg mice have a reduction in *Pomc*-positive cells (L). (**M-O**) Similarly, *Mash1* mRNA is detected within the Arc HVZ of control mice (M), diffusely expressed within the Arc of *Rbpj*κ cKO mice (N), and reduced in NICD Tg mice (O). (**P**,**R**) TUNEL staining reveals no cell death within the developing Arc in control (P) and NICD Tg mice (R). (**Q**) By contrast, TUNEL-positive cells within ventricular zone are found in *Rbpj*κ cKO mice (dashed lines). Scale bar: 50 μm.

The regulation of progenitor cells through apoptosis is an important developmental event that occurs during the transition from neural progenitor to differentiated neuronal subtype (Blaschke et al., 1998; Price et al., 1997). In fact, loss of Notch signaling through *Hes1* can induce apoptosis (Jensen et al., 2000; Raetzman et al., 2007). We therefore used TUNEL to assay for cell death to determine whether Notch/Rbpjκ may be affecting progenitor survival within the Arc of *Rbpj*κ cKO and NICD Tg mice. We found that there are no TUNEL-positive cells within the Arc of control (Fig. 3P) or NICD Tg mice (Fig. 3R), whereas TUNEL-positive cells aberrantly line the ventricular zone within the Arc of *Rbpj*κ cKO mice (Fig. 3Q) at E13.5. Therefore, our data suggest that Notch/Rbpjκ signaling is necessary for progenitor survival in the Arc.

Notch signaling is required for proper formation of the hypothalamic ventricular zone

We have shown that progenitors within the Arc HVZ express Notch receptors and downstream targets at E13.5 (Figs 1, 2) and that Notch/Rbpjκ affects proliferation, differentiation and cell survival of Arc progenitors at E13.5. In order to determine how loss of Notch/Rbpjκ and persistent expression of NICD would affect Arc progenitors later in embryonic development, we examined the HVZ of *Rbpj*κ cKO and NICD Tg mice at E18.5. We found that loss of Notch/Rbpjκ causes a complete loss of the HVZ (dashed box; Fig. 4B), which correlates with the cell death observed at E13.5 in this region. By contrast, NICD Tg mice display an apparent expansion of HVZ cells (Fig. 4C) compared with controls (Fig. 4A). In parallel, Sox2 immunostaining reveals a loss of Sox2-immunopositive progenitors lining the third ventricle in *Rbpj*κ cKO mice (Fig. 4E) compared with controls (Fig. 4D). By contrast, persistent expression of Notch results in an expanded HVZ comprising Sox2-immunopositive cells in NICD Tg mice (Fig. 4F).

To confirm that the changes observed with Sox2 expression were reflective of neural progenitors, we examined nestin expression and found that the nestin-immunopositive cells that line the third ventricle are lost in *Rbpj*κ cKO mice (Fig. 4H) compared with controls (Fig. 4G), whereas NICD Tg mice show an expansion of nestin-immunopositive cells localized throughout HVZ (Fig. 4I). Similarly, mRNA expression of the hypothalamic-specific progenitor marker *Rax* is lost in *Rbpj*κ cKO mice (Fig. 4K), and expanded in NICD Tg mice, specifically throughout the ventral-most region (Fig. 4L) compared with controls (Fig. 4J). Taken together, these results suggest that Notch/Rbpjκ signaling is required for survival and maintenance of progenitors as well as progenitor number in the Arc HVZ.

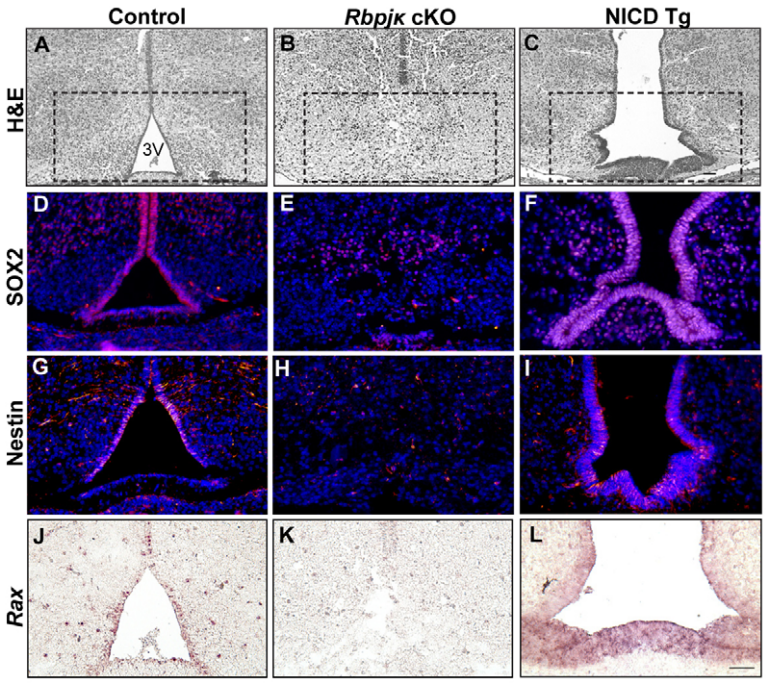

Fig. 4. Notch signaling is required for proper formation of the Arc hypothalamic ventricular zone at E18.5. (**A**) Hematoxylin and Eosin staining on coronal sections show the third ventricle (3V) and hypothalamic ventricular zone (HVZ; outlined). (**B**,**C**) *Rbpjκ* cKO mice show a loss of the Arc HVZ (B), whereas NICD Tg mice have an expanded Arc HVZ (C). (**D-F**) Sox2 immunostaining shows a loss of Sox2-immunopositive cells lining the 3V in *Rbpjκ* cKO mice (E) compared with controls (D), whereas NICD Tg animals have an expanded Arc HVZ comprising Sox2-immunopositive cells (F). (**G-I**) Nestin-immunopositive cells label neuronal progenitors along HVZ (G); this population is lost in *Rbpjκ* cKO mice (H) and expanded in NICD Tg mice (I). (**J**) *In situ* hybridization using the hypothalamic progenitor marker *Rax* labels cells within the Arc HVZ in controls animals. (**K**,**L**) *Rbpjκ* cKO mice show few *Rax*-positive cells (K), whereas NICD Tg animals have an expanded *Rax*-positive region (L). Scale bar: 50 μm.

Persistent expression of Notch holds progenitor cells in the hypothalamic ventricular zone

We found that persistently activated Notch increases the population of progenitor cells found within the HVZ at E18.5 (Fig. 4). In order to determine whether the increased HVZ cell population is due to a continuing increase in HVZ cell genesis, we examined the expression of the proliferation marker Ki67. We found that Ki67-immunopositive cells were not present in control (Fig. 5A) or NICD Tg mice (Fig. 5B), indicating activated Notch is not sufficient to induce proliferation at postnatal day 1 (P1).

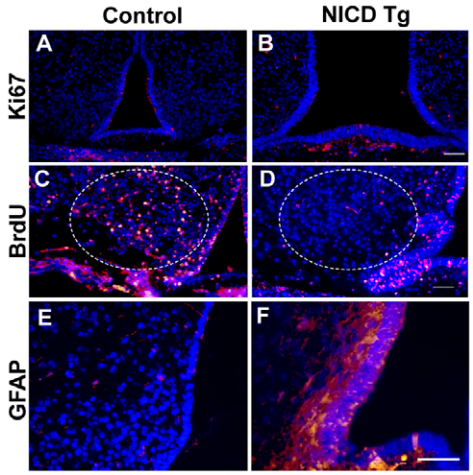

Fig. 5. Hypothalamic cells born at E12.5 remain in the hypothalamic ventricular zone when Notch is persistently expressed. (**A**,**B**) Coronal sections of the Arc at postnatal day 1 (P1) show that cells within the HVZ are not Ki67 immunopositive in control (A) or NICD Tg (B) mice, indicating that these cells are not proliferating at P1. BrdU immunohistochemistry was performed on mice at P1 that were injected with BrdU at E12.5. (**C**,**D**) BrdU-immunopositive cells are present within the HVZ and Arc (outlined) in control animals (C), but not in the Arc of NICD Tg mice (D). (**E**,**F**) Arc HVZ cells of NICD Tg mice are GFAP immunopositive (F), whereas control Arc HVZ cells do not express GFAP-immunopositive cells at this age (E). Scale bar: 50 μm.

An alternate possibility is that the increase in cells within the HVZ in NICD Tg mice is due to a build up of progenitor cells that have not migrated to hypothalamic nuclei. In order to test this hypothesis, we injected control and NICD Tg mice with BrdU, a thymidine analog that is incorporated into newly synthesized DNA of replicating cells (Kuhn et al., 1996) at E12.5. We then performed BrdU-immunohistochemistry at P1 to determine the location of cells born at E12.5, the peak of hypothalamic neurogenesis (Ishii and Bouret, 2012). We found that BrdU-immunopositive cells are present within HVZ and the Arc (dashed circle) in control mice (Fig. 5C), but are present only within the HVZ in NICD Tg mice (Fig. 5D). Taken together with data showing increased Arc proliferation in NICD Tg mice at E13.5 (Fig. 3I), these results indicate that the increase in the ventral HVZ population is due to both proliferation during early embryonic development, as well as progenitor cells being retained in the HVZ that do not differentiate into neurons.

In addition to its role in progenitor maintenance, Notch signaling is also known to promote glial differentiation in other tissues (Furukawa et al., 2000; Gaiano et al., 2000; Gaiano and Fishell, 2002). We therefore examined the expression of glial fibrillary acidic protein (GFAP) to determine whether the cells within the HVZ in NICD Tg mice adopted a glial fate. We found that many cells along the ependymal surface within NICD Tg mice are GFAP immunopositive (Fig. 5F), compared with HVZ cells in controls, which do not yet express GFAP at this stage of development (Fig. 5E). Our data indicate that persistent Notch expression leads to accumulation of progenitor cells within the developing Arc HVZ, some of which aberrantly assume a glial fate.

Notch signaling is required for proper differentiation of arcuate neurons

By E18.5, a subset of progenitors within HVZ have migrated to the Arc and differentiated into neuronal subtypes (McNay et al., 2006; Pelling et al., 2011; Yee et al., 2009). Given that loss and gain of

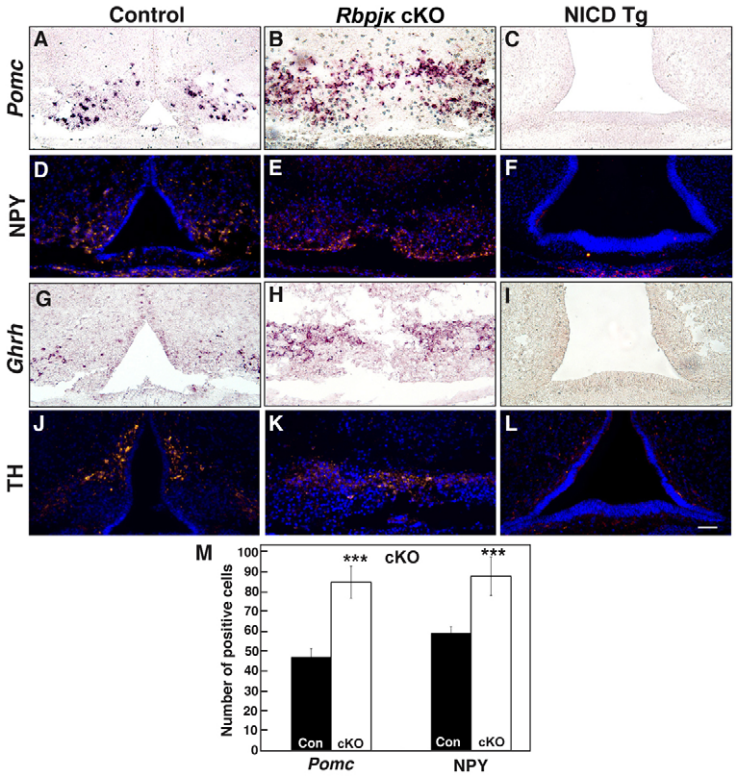

Fig. 6. Rbpjκ-dependent Notch signaling is required for proper differentiation of Arc neurons at E18.5. (**A**) *In situ* hybridization on coronal sections shows *Pomc*-positive cells in the Arc of controls. (**B**,**C**) *Rbpjκ* cKO mice have significantly more *Pomc*-positive neurons (B), whereas NICD Tg mice show no differentiation of *Pomc*-positive cells (C). (**D-F**) NPY-immunopositive cells are present in the Arc of control mice (D), are significantly increased in number ($P<0.001$; E) in *Rbpjκ* cKO animals and are not detected in NICD Tg animals (F). (**G-I**) *In situ* hybridization reveals *Ghrh*-positive cells in the Arc of control animals (G), an apparent increase in the number of these cells in *Rbpjκ* cKO mice (H) and no differentiation of these cells in NICD Tg mice (I). (**J-L**) Similarly, TH-immunopositive cells are present in the Arc of control mice (J) and appear increased in *Rbpjκ* cKO animals (K), whereas few cells are TH-immunopositive in NICD Tg mice (L). (**M**) Quantification of A-F (***$P<0.001$).

Notch dramatically affects Arc progenitors at E18.5, we examined the differentiation of Arc neurons at the same developmental time point. We found that loss of Notch increased the number of *Pomc*-positive cells in the Arc (84.7±8.1, $P<0.001$; Fig. 6B,M) compared with controls (47.1±4.2; Fig. 6A,M). By contrast, persistent activation of Notch results in absence of differentiated *Pomc* neurons (Fig. 6C). The number of NPY-immunopositive cells was also increased in *Rbpjκ* cKO mice (87.7±9.7, $P<0.001$; Fig. 6E,M) compared with controls (59.1±3.2; Fig. 6D,M), whereas NICD Tg mice show no NPY-immunopositive cells within the presumptive Arc. *Ghrh*-positive and TH-immunopositive neurons are also increased in *Rbpjκ* cKO mice (Fig. 6H,K) compared with controls (Fig. 6G,J), whereas NICD Tg mice have no apparent differentiation of *Ghrh*-positive or TH-immunopositive neurons (Fig. 6I,L). These data indicate that Notch/Rbpjκ signaling regulates Arc neuron number and that it must be turned off for Arc neuron differentiation to occur.

Body size is altered in mice with loss and gain of Notch signaling in the ventral hypothalamus

We found that loss of Notch/Rbpjκ signaling and persistent Notch expression *in vivo* affects differentiation of *Pomc* and NPY neurons, as well as *Ghrh* neurons, which regulate feeding and body size, respectively. In order to determine the long-term physiological effects of disrupted Arc formation, we measured postnatal body weight of cKO and Tg mice and their controls for 8 weeks (Fig. 7). We found that *Rbpjκ* cKO mice weigh significantly more than *Rbpjκ* cKO controls at week 7 and week 8 (Fig. 7C,E). Additionally, NICD Tg mice appear to have a reduced length and weigh significantly less than their controls at weeks 3, 4 and 5 (Fig. 7B,E). However, despite their apparent reduction in body length, by week 7 NICD Tg mice have similar body weight compared with controls (Fig. 7D,E). These results suggest that manipulations in Notch signaling that result in changes in differentiation of neurons that control body size and metabolism have significant consequences on body weight *in vivo*.

DISCUSSION

Development of the hypothalamus arises from coordinated signaling that induces patterning genes, which allow the formation of neuronal clusters with distinct function. The development of the Arc can be divided into three main events during embryogenesis: hypothalamic regionalization (before E10.5), specification and differentiation of Arc progenitors (E10.5-15.5), and organization of hypothalamic nuclei (E16.5-18.5) (Caqueret et al., 2005; McClellan et al., 2006; Shimada and Nakamura, 1973). Once regionalized, a subset of multipotent *Sox2* and *Rax* Arc progenitors must exit the cell cycle and undergo neuronal differentiation (Lu et al., 2013). The expression of bHLH proneural genes such as *Mash1* is necessary to promote differentiation of Arc neurons into neuronal subtypes (Kim et al., 2008; McNay et al., 2006) and generate a distinct nucleus.

Although certain factors necessary for Arc development, such as *Rax* and *Mash1*, have been identified, how these signals are integrated to direct differentiation of Arc neurons and subsequent Arc nucleus formation is unknown. The current study examines the possibility that Notch signaling is the crucial mediator of progenitor cell cycle exit and differentiation. We found that Notch/Rbpjκ signaling, acting through activation of *Hes1*, maintains Arc progenitor number and survival, inhibits Arc neuronal differentiation and is sufficient induce precocious astroglial differentiation within the Arc ventricular zone (see Fig. 8).

An important finding is that loss of Rbpjκ resulted in loss of *Hes1* but not of *Hes5* (Fig. 2), suggesting that *Hes5* expression may be independent of Notch/Rbpjκ signaling in the Arc. It is well established that *Hes1* expression can be regulated by Notch/Rbpjκ-independent signaling before E8.5 (Hatakeyama et al., 2004; Kageyama et al., 2005). Additionally, Rbpjκ-independent expression of Notch factors, including Hes genes, can be mediated by members of the BMP (Dahlqvist et al., 2003; Itoh et al., 2004), TGFβ (Blokzijl et al., 2003; Klüppel and Wrana, 2005; Ross and

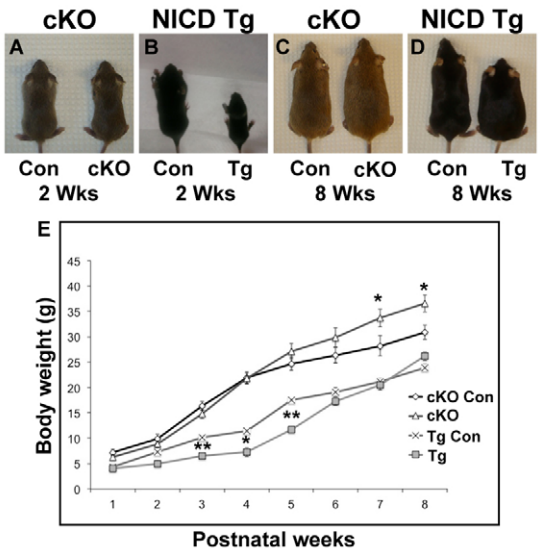

Fig. 7. Body size is altered in mice with loss of Rbpjκ-dependent Notch signaling, as well as in mice with persistent Notch expression in the Arc. (**A**,**B**) *Rbpjκ* cKO mice appear normal in size and weight compared with controls at 2 postnatal weeks (2 wks; A), whereas NICD Tg mice are smaller in size (B). (**C**,**D**) At 8 weeks, *Rbpjκ* cKO (C) and NICD Tg (D) mice have increased body weight compared with littermate controls, yet NICD Tg mice appear shorter in length. Male and female mice were weighed once per week until 8 postnatal weeks; female mice are pictured. (**E**) *Rbpjκ* cKO mice begin increasing in body weight at week 5 and weigh significantly more than controls at week 7 ($P<0.05$) and week 8 ($P<0.05$). NICD Tg mice have significantly lower body weight at week 3 (**$P<0.01$), week 4 (*$P<0.05$) and week 5 (**$P<0.01$).

Kadesch, 2001), WNT/β-catenin (Axelrod et al., 1996; Hayward et al., 2005) and Shh (Wall et al., 2009) pathways throughout embryonic development. However, only a few studies have shown that *Hes5* can be modulated by Notch/Rbpjκ-independent signaling occurring at two distinct time points: before E8.5 (Donoviel et al., 1999; Hitoshi et al., 2011) and during adult neurogenesis (Matsuda et al., 2012). Our study is the first to show that *Hes5* expression may be regulated by Rbpjκ-independent signaling during embryonic neurogenesis *in vivo*. Given that other Notch targets can be modulated by BMP, TGFβ, WNT/β-catenin and Shh, it is possible that the *Hes5* expression observed in the absence of Rbpjκ may be mediated by one of these pathways in the developing Arc.

Our results suggest that Notch/Rbpjκ signaling mediated by *Hes1* affects maintenance of the Arc progenitor pool by repressing *Mash1*. In the current study, loss of *Hes1* results in robust *Mash1* expression, cell proliferation and increased *Pomc* differentiation (Fig. 3). The antagonistic expression and function of *Hes1* and *Mash1* are well established in other contexts. *Hes1* is known to bind to the *Mash1* promoter (Chen et al., 1997) to prevent its transcriptional activation (Sasai et al., 1992). In fact, repression of proneural genes such as *Mash1* is the proposed mechanism of *Hes1*-induced inhibition of neuronal differentiation (Kageyama et al., 1997; Kageyama et al., 2005; Schuurmans and Guillemot, 2002). *Mash1*, which is normally antagonized by *Hes1*, encourages progenitor proliferation (Castro et al., 2011) and rapidly induces cell cycle exit leading to neuronal commitment (Casarosa et al., 1999). This suggests that, in the current study, loss of *Hes1* and increased *Mash1* leads to increased cell proliferation and Arc differentiation in *Rbpjκ* cKO mice. Our results additionally show that persistent Notch activation results in reduced *Mash1* expression and reduced *Pomc* differentiation in NICD Tg mice (Fig. 3), confirming that Notch/Rbpjκ is both required and sufficient within an Arc progenitor to repress *Mash1* and maintain progenitor fate.

We also found that *Rbpjκ* cKO mice have aberrant cell death within the Arc at E13.5. The observed cell death within the Arc ventricular zone could be due to *Hes1* loss. Importantly, loss of *Hes1* reduces the self-renewing ability of telencephalic progenitors and accelerates progenitors towards the neuronal lineage, resulting in an increased number of committed progenitors that follow an apoptotic fate (Nakamura et al., 2000). Although there is observable Arc progenitor apoptosis at E13.5, *Rbpjκ* cKO mice still maintain a population of Sox2-positive progenitors, perhaps owing to the sustained *Hes5* expression observed in the Arc ventricular zone. Therefore, in our model of Rbpjκ loss, *Hes5* acting independently of Rbpjκ may maintain a subset of Arc progenitors that are proliferating and differentiating due to increased *Mash1* activity, and the absence of *Hes1* may also contribute to some progenitor cell death at E13.5. By contrast, conditional deletion of Rbpjκ from the telencephalon and subsequent loss of both *Hes1* and *Hes5* results in complete depletion of ventricular zone progenitors, coincident with a reduction in telencephalon size and accelerated neural commitment (Imayoshi et al., 2010). Therefore, our results suggest a novel Rbpjκ-independent role of *Hes5* in maintaining the Arc progenitor population that is not observed in other brain regions. Notably, by E18.5 *Rbpjκ* cKO mice no longer have progenitor cells lining the ventricular zone (Fig. 4). Loss of progenitors probably results from a combination of progenitor death observed at E13.5, as well as increased differentiation of progenitors to *Pomc*, NPY, TH and *Ghrh* neurons (Fig. 6).

We demonstrate that persistent expression of Notch leads to robust and sustained progenitor build up within the ventricular

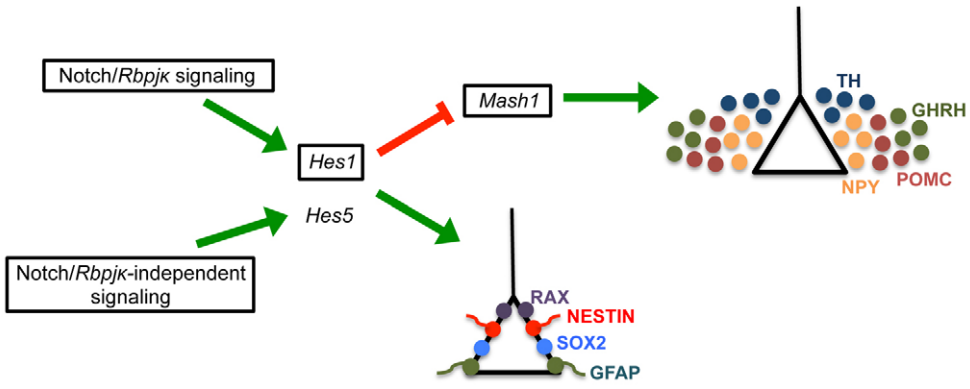

Fig. 8. Notch/Rbpjκ signaling regulates progenitor maintenance and differentiation of hypothalamic Arc neurons through *Hes1* activation and repression of *Mash1*.

zone (Figs 4, 5) and subsequent lack of Arc neuron differentiation at E18.5 (Fig. 6). Previous reports have also observed that both overexpression of *Hes1* and activated Notch signaling inhibits neuronal differentiation within the cortex (Ishibashi et al., 1994). We also found that the expanded progenitor zone observed in NICD Tg mice is coincident with expanded Sox2-immunopositive and nestin-immunopositive cells. Sox2 is an important factor in inhibiting cell differentiation and maintaining stem cell character (Episkopou, 2005). In fact, the Notch intracellular domain (NICD), which is constitutively activated in the NICD Tg model reported here, is able to activate directly the transcription of the *Sox2* promoter (Ehm et al., 2010). Notch overexpression can also activate the nestin promoter (Shih and Holland, 2006), which supports the observation of increased nestin-immunopositive cells in NICD Tg mice (Fig. 4). Taken together, our data suggest that persistent Notch signaling directly maintains cells in a progenitor state and does not allow for proper differentiation into Arc neurons.

We show that *Pomc*, NPY and *Ghrh* neuron number relies on regulated levels of Notch signaling and that alterations in Notch signaling affect postnatal body weight and size. The reduced body length and body weight observed in NICD Tg mice may be in part due to the absence of *Ghrh* in the Arc (Fig. 6), which controls proper growth, as well as additional disruption of the hypothalamic-pituitary axis. *Rbpjκ* cKO mice have significantly increased body weight and increased Arc neurons, which likely contributes to the changes observed in postnatal body weight. Notably, *Nkx2.1* is also expressed during early development within the thyroid gland and lung (Lazzaro et al., 1991); therefore, manipulations of Notch/Rbpjκ in *Nkx2.1*-positive regions outside the hypothalamus may contribute to the body weight phenotype observed.

Our study establishes that Notch/Rbpjκ signaling within the developing Arc is crucial to establish the balance of progenitors and differentiated cells, including glia. Progenitor cells along the ventricular zone give rise to both neurons during embryonic development, as well as to astrocytes after birth (Qian et al., 2000). Notch/Rbpjκ signaling has been implicated in astrogliogenesis through demonstration of direct binding of NICD/Rbpjκ complex to the *Gfap* gene *in vitro* (Ge et al., 2002). Additionally, suppression of neurogenic bHLH factors, such as *Mash1*, is necessary for astrogliogenesis in the cortex (Nieto et al., 2001). By contrast, activated *Notch1* promotes precocious astroglial differentiation *in vitro* (Kohyama et al., 2005). We show that persistent expression of Notch *in vivo* also results in precocious astroglial differentiation within the HVZ at P1 (Fig. 5). These data are the first to reveal that tightly controlled Notch signaling instructs proper timing of astroglial differentiation within the Arc *in vivo*.

We demonstrate that Notch/Rbpjκ signaling is important not only for astroglial differentiation within the Arc, but also contributes to maintenance of Arc progenitors and is required for restraining Arc neuron differentiation at the expense of progenitors. During development, Arc POMC and NPY neurons project to hypothalamic nuclei such as the paraventricular nucleus, establishing complex circuitry responsible for regulating feeding behavior and energy metabolism. Identifying molecular pathways, such as Notch signaling, that control development of these neurons is crucial to understanding dysregulation of feeding and energy balance that occurs in metabolic disease. Recent studies have shown that the hypothalamic proliferative zone (HPZ) can undergo postnatal neurogenesis (Lee and Blackshaw, 2012; McNay et al., 2012; Shimogori et al., 2010) and is responsive to diet. In the adult mouse, the HPZ is enriched with Notch factors and future studies could use this mouse model to explore the effect of Notch/Rbpjκ signaling on the function of these neurogenic cells during postnatal development and adulthood.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
P.K.A. and L.T.R. designed the study, interpreted the data and wrote the manuscript; P.K.A., G.T.N. and L.X. performed the experiments.

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