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The Notch effector gene *Hes1* regulates migration of hypothalamic neurons, neuropeptide content and axon targeting to the pituitary☆

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ABSTRACT

Proper development of the hypothalamic-pituitary axis requires precise neuronal signaling to establish a network that regulates homeostasis. The developing hypothalamus and pituitary utilize similar signaling pathways for differentiation in embryonic development. The Notch signaling effector gene *Hes1* is present in the developing hypothalamus and pituitary and is required for proper formation of the pituitary, which contains axons of arginine vasopressin (AVP) neurons from the hypothalamic paraventricular nucleus (PVN) and supraoptic nucleus (SON). We hypothesized that *Hes1* is necessary for the generation, placement and projection of AVP neurons. We found that *Hes1* null mice show no significant difference in cell proliferation or death in the developing diencephalon at embryonic day 10.5 (e10.5) or e11.5. By e16.5, AVP cell bodies are formed in the SON and PVN, but are abnormally placed, suggesting that *Hes1* may be necessary for the migration of AVP neurons. GAD67 immunoreactivity is ectopically expressed in *Hes1* null mice, which may contribute to cell body misplacement. Additionally, at e18.5 *Hes1* null mice show continued misplacement of AVP cell bodies in the PVN and SON and additionally exhibit abnormal axonal projection. Using mass spectrometry to characterize peptide content, we found that *Hes1* null pituitaries have aberrant somatostatin (SS) peptide, which correlates with abnormal SS cells in the pituitary and misplaced SS axon tracts at e18.5. Our results indicate that Notch signaling facilitates the migration and guidance of hypothalamic neurons, as well as neuropeptide content.

Introduction

The hypothalamic-pituitary axis (HP) is a master controller of endocrine processes, such as metabolism, growth and reproduction. The neuroendocrine hypothalamus contains nuclei with two distinct neuronal populations: magnocellular and parvocellular. Magnocellular neurons located in the paraventricular nuclei (PVN) and the supraoptic nuclei (SON) release arginine vasopressin (AVP) and oxytocin (OT) from their axonal terminals within the posterior lobe (PL) of the pituitary. OT acts on the periphery by inducing lactation and uterine contraction. In the central nervous system, OT facilitates social behavior, including parental care and bonding (Lim et al., 2005; Lim and Young, 2006). AVP also exerts central effects on behavior, including aggression (Heinrichs et al., 2009). AVP released from the PL is crucial to nutrient reabsorption and regulating the body's response to stress.

The hypothalamic anterior periventricular (aPV) nucleus contains parvocellular somatostatin (SS)-releasing neurons, and growth hormone-releasing hormone (GHRH) neurons are found in the arcuate nucleus (ARN). GHRH activates secretion of somatotropes in the anterior lobe of the pituitary (AL). Somatotropes secrete growth hormone (GH) and promote linear growth and metabolism, and SS inhibits the secretion of GH to regulate this process.

Magnocellular neurons, parvocellular neurons and pituitary cells demonstrate striking temporal and spatial coordination in events that regulate their differentiation. These cells form from the embryonic basal plate, with the anterior ridge generating the AL, and the adjacent region developing into the hypothalamus and the PL (Couly and Le Douarin, 1987, 1988; Kawamura and Kikuyama, 1995; Kouki et al., 2001). In the mouse, hypothalamic neurons are generated between e10.5 and e12.5 from the proliferative neuroepithelium of the third ventricle (Shimada and Nakamura, 1973). Several signaling pathways and transcription factors have been implicated in formation of these neurons in the PVN and SON (Michaud et al., 1998, 2000; Hosoya et al., 2001). From the neuroepithelium, neurons migrate laterally to form the SON, or medially to form the PVN and aPV by e14.5 (Altman and Bayer, 1978, 1979; Bayer and Altman, 1987). Various guidance cues, specifically, members of the Netrin, Slit/Robo and Semaphorin/Plexin/Neuropilin families are expressed in and around the developing PVN and SON, and have been implicated in cell migration (Deiner and Sretavan, 1999; Xu and Fan, 2007, 2008). GABAergic neurons have also been implicated in proper boundary formation of the hypothalamic PVN and ventromedial nucleus (VMN) during development

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through $GABA_A$ and $GABA_B$ receptors (Dellovade et al., 2001; Davis et al., 2002; McClellan et al., 2008, 2010).

Although initial studies have begun to identify factors that are generally required for formation and migration of hypothalamic neurons, the mechanisms regulating specific neuroendocrine cell development remain unclear. We hypothesize that the Notch signaling pathway is required for proper formation, migration and projection of AVP and SS neurons to the pituitary.

Notch signaling is an evolutionarily conserved mechanism that guides progenitor maintenance and cell specification in the developing nervous system. In the cerebellum, loss of *Notch1* results in the premature differentiation of neurons at the expense of undifferentiated cells (Lutolf et al., 2002), and persistent activation of *Notch2* maintains precursors in a proliferative state (Solecki et al., 2001). Similarly, *Hes1* and *Hes3* double null mice show premature neuron formation in the mid/hind brain and subsequent loss of midbrain and anterior hindbrain structures (Hirata et al., 2001). Additionally, overexpression of *Hes1* in the telencephalon inhibits neuronal differentiation (Ohtsuka et al., 2001).

Previous studies have delineated the importance of the Notch pathway in early specification events that regulate cell fate in the developing HP. The Notch effector gene *Hes1* is spatially and temporally restricted to the developing diencephalon and pituitary during development, and its expression must be silenced for pituitary cell differentiation to occur (Zhu et al., 2006; Kita et al., 2007; Raetzman et al., 2007). *Hes1* null mice survive until embryonic day 18.5 (e18.5) and show a reduction in PL size (Raetzman et al., 2007; Himes and Raetzman, 2009), which contains terminal axons of AVP and OT neurons. Previous studies have shown that the development of the ventral diencephalon (VD) also relies on *Hes1,* with *Hes1* null mice displaying hypoplastic phenotypes of the developing pituitary and diencephalon (Akimoto et al., 2010). Importantly, there is emerging evidence implicating *Hes1* in cell migration and cell placement in the developing pituitary (Himes and Raetzman, 2009). Given that the primordial pituitary and hypothalamus share signaling pathways that generate differentiation and migration cues, it is likely that *Hes1* may play a role in the development and placement of endocrine neurons within the hypothalamus.

In order to address the extent that Notch signaling is required for functional neuronal development within the HP, we analyzed *Hes1* null mice and control littermates at various stages of embryonic development. We found no significant difference in the number of proliferating cells or cell death in the VD. AVP cell bodies are specified in *Hes1* null mice and aberrantly placed, which correlates with ectopic GAD67 expression within these regions. We found abnormal projections of AVP-positive axons to the PL in *Hes1* null mice. Additionally, we utilized mass spectrometry-based peptidomics (Li and Sweedler, 2008) to screen for peptide alterations in *Hes1* null pituitaries at e18.5. We uncovered AVP-related products in *Hes1* null mice as well as unexpected SS peptide content. Further analyses showed a reduction of SS-positive cells in the aPV, aberrant SS-positive axons, and abnormal SS-positive expression in the PL of *Hes1* null mice. The alterations in peptide content, and axon pathfinding to and termination in the pituitaries of *Hes1* null mice indicate that Notch signaling facilitates the formation of AVP and SS neurons, guidance of hypothalamic axons to the pituitary, and neuropeptide processing.

Materials and methods

Animals

Hes1 mutant mice were previously generated by replacing the first 3 exons with a neomycin-resistance cassette (Ishibashi et al., 1994). Breeding colonies were generated at the University of Illinois at Urbana-Champaign (UIUC) and maintained on a mixed genetic background of C57BL/6 J and CD1 mice. All animal procedures were approved by the UIUC Institutional Animal Care and Use Committee. Heterozygous males and females were mated to generate mixed genotype litters, which were genotyped as previously described (Jensen et al., 2000). Embryos were collected at e10.5, e11.5, e16.5 and e18.5, and either prepared for immunohistochemistry or peptide extraction at e18.5.

Immunohistochemistry

After collection, embryos were fixed in formaldehyde, embedded in paraffin and processed for immunohistochemistry as previously described (Monahan et al., 2009). Primary antibodies were as follows: rabbit anti-arginine vasopressin (Abcam, Cambridge, MA USA 1:500; Fitzgerald Industries, Acton, MA, USA 1:500), rabbit anti-somatostatin-28 antibodies (Bachem, Torrance, CA, USA 1:500; Millipore, Billerica, MA, USA 1:100), and rabbit anti-phosphohistone H3 (PH3) (Millipore, 1:300). Species specific secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA, USA). Nickel (II) sulfate 3,3-diaminobenzidine (NiDAB) immunohistochemistry, adapted from Kramer et al. (2005) was performed on parasagittal sections embedded at a 30° horizontal plane. Subsequent to primary and secondary antibody incubation, a Vectastain kit (Vector Laboratories, Burlingame, CA, USA) diluted in PBS was used. Slides were then equilibrated in 0.175 M sodium acetate. Next, NiDAB solution (2.5% nickel II sulfate, 2% DAB, 0.02% H_2O_2 in sodium acetate) was applied to the slides for 20–30 min at room temperature. Slides were then washed in 0.175 M sodium acetate 2 times for 5 min and PBS 2 times for 5 min. Finally, slides were counterstained with methyl green, dehydrated and mounted with Permount (Fisher). Samples were visualized at a 100×, 200× and 400× magnification using a Leica DM 2560 microscope. Images were taken with a Retiga 2000 camera using Q Capture Pro software (QImaging, Surrey, BC, Canada) and processed with Adobe Photoshop, version 11.0.2 (Adobe Systems Incorporated, San Jose, CA, USA).

PH3-positive and AVP-positive cell quantification

Midsagittal sections of the VD taken from *Hes1* null embryos and littermate controls at e10.5 and e11.5 were immunostained with PH3 and DAPI as described. Sections from the mid-PVN and mid-SON of *Hes1* null embryos and littermate controls at e16.5 were immunostained with AVP as described. Images were taken at 200× magnification. The number of solid PH3-positive cells in the VD were counted and divided by the total number of DAPI-positive cells in the VD to obtain the proportion of PH3-positive cells in the VD. The numbers of AVP-positive cells were counted in the mid-PVN and mid-SON. For PH3 and AVP cell counts, 4 sections per animal were analyzed and the average proportion of PH3-positive or AVP-positive cells was compared between three *Hes1* null embryos and three littermate controls. These values were tested for statistical significance using a Student's *t* test (SAS 9.1 software; SAS Institute, Cary, NC, USA).

Quantitative real time-PCR

Whole brains were dissected at e16.5 and snap frozen in ethanol. RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed to create cDNA from 0.5 g of isolated mRNA template. qRT-PCR was performed using cDNA with primers for *Avp*, *Gad1* and *Gapdh*. The primer sequences are as follows: *Avp* forward 5′ CTC TCC GCT TGT TTC CTG AG 3′, *Avp* reverse 5′ CTC TTG GGC AGT TCT GGA AG 3′, *Gad1* forward 5′ CTC CAA GGA TGC AAC CAG AT 3′, *Gad1* reverse 5′ CTG GAA GAG GTA GCC TGC AC 3′, *Gapdh* forward 5′ GGT GAG GCC GGT GCT GAG TAT G 3′, and *Gapdh* reverse 5′ GAC CCG TTT GGC TCC ACC CTT C 3′. Samples were run and analyzed on Bio-Rad iCycler IQ (Bio-Rad Laboratories, Hercules, CA, USA). The PCR conditions for all primer sets used were