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Rbpj-κ mediated Notch signaling plays a critical role in development of hypothalamic Kisspeptin neurons

Matthew J. Biehl, Lori T. Raetzman *

Department of Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

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ABSTRACT

The mammalian arcuate nucleus (ARC) houses neurons critical for energy homeostasis and sexual maturation. Proopiomelanocortin (POMC) and Neuropeptide Y (NPY) neurons function to balance energy intake and Kisspeptin neurons are critical for the onset of puberty and reproductive function. While the physiological roles of these neurons have been well established, their development remains unclear. We have previously shown that Notch signaling plays an important role in cell fate within the ARC of mice. Active Notch signaling prevented neural progenitors from differentiating into feeding circuit neurons, whereas conditional loss of Notch signaling lead to a premature differentiation of these neurons. Presently, we hypothesized that Kisspeptin neurons would similarly be affected by Notch manipulation. To address this, we utilized mice with a conditional deletion of the Notch signaling co-factor *Rbpj-κ* (*Rbpj* cKO), or mice persistently expressing the *Notch1* intracellular domain (NICD tg) within *Nkx2.1* expressing cells of the developing hypothalamus. Interestingly, we found that in both models, a lack of Kisspeptin neurons are observed. This suggests that Notch signaling must be properly titrated for formation of Kisspeptin neurons. These results led us to hypothesize that Kisspeptin neurons of the ARC may arise from a different lineage of intermediate progenitors than NPY neurons and that Notch was responsible for the fate choice between these neurons. To determine if Kisspeptin neurons of the ARC differentiate similarly through a *Pomc* intermediate, we utilized a genetic model expressing the tdTomato fluorescent protein in all cells that have ever expressed *Pomc*. We observed some Kisspeptin expressing neurons labeled with the *Pomc* reporter similar to NPY neurons, suggesting that these distinct neurons can arise from a common progenitor. Finally, we hypothesized that temporal differences leading to premature depletion of progenitors in cKO mice lead to our observed phenotype. Using a BrdU birthdating paradigm, we determined the percentage of NPY and Kisspeptin neurons born on embryonic days 11.5, 12.5, and 13.5. We found no difference in the timing of differentiation of either neuronal subtype, with a majority occurring at e11.5. Taken together, our findings suggest that active Notch signaling is an important molecular switch involved in instructing subpopulations of progenitor cells to differentiate into Kisspeptin neurons.

1. Introduction

The hypothalamus is a key mediator of many homeostatic functions in mammalian physiology (Brooks, 1988). In humans and rodents, it has been linked to regulation of water balance, energy balance, thermal regulation, reproductive function, body size, stress and others (Epelbaum, 1992; McCann et al., 1994; Rothwell, 1994; Becu-Villalobos and Libertun, 1995; Stratakis and Chrousos, 1995; Bing et al., 1996). In order to perform such a wide array of functions, this region of the brain is separated into subsets of neurons, called nuclei, which signal through distinct neuropeptides produced by specialized neuronal subtypes (Swaab et al., 1993). One nucleus, the arcuate nucleus (ARC), has classically been characterized as the "feeding center" of the brain (Swaab et al., 1993). This function is carried out by the antagonistic actions of anorexigenic Proopiomelanocortin (POMC) and orexigenic Neuropeptide-Y (NPY)/Agouti-related Peptide (AgRP) neurons (Cowley et al., 2001; Luquet et al., 2005). These neuronal subsets interact not only with each other, but send projections to other areas of the brain (Wang et al., 2015). Aside from regulating energy homeostasis, the ARC also has a population of Kisspeptin expressing neurons (Gottsch et al., 2004). Kisspeptin neurons have been suggested to play a role in puberty onset and reproductive function by communicating with Gonadotropin-releasing hormone (GnRH) neurons and acting as a pulse generator for the release of

* Correspondence to: University of Illinois at Urbana-Champaign, 542 Burrill Hall, 407 South Goodwin Avenue, Urbana, IL 61801, USA. Fax: +1 217 3331133.
E-mail address: raetzman@life.illinois.edu (L.T. Raetzman).

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luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Irwig et al., 2004; Li et al., 2009). There are at least two other neuron populations found within the ARC as well, one expressing Growth Hormone Releasing Hormone (GHRH) and the other Tyrosine Hydroxylase (TH) (Balan et al., 1996; Romero and Phelps, 1997). Despite the differences in peptide content and function, ARC neurons likely all arise from a common pool of progenitor cells in the developing diencephalon (Yee et al., 2009). The exact molecular pathways that allow a progenitor to create these neurons of distinct fates are still largely unknown. Importantly, genetic disorders that affect early development of the hypothalamus, can result in life-long deficiencies that persist into adulthood. For example, many of the phenotypes observed in Prader–Willi patients are regulated by neurons found within the ARC (Ge et al., 2002). Namely, hyperphagy may be modulated by the POMC and NPY expressing population of neurons within the ARC, whereas the Kisspeptins of the ARC may be contributing to the observed infertility and hypogonadism. This example highlights the importance of understanding neural development and the genes responsible for differentiation of each neuronal subtype within the hypothalamus.

Neurogenesis and development of the ARC begins as early as embryonic day 10.5 (e10.5) (Shimada and Nakamura, 1973; McNay et al., 2006). Previous reports have shown that in the mouse a great deal of the neurogenesis specifically within the ARC occurs in a very narrow window, and by e16.5 all the cellular subtypes within the ARC are present (Alvarez-Bolado et al., 2012; Ishii and Bouret, 2012). Progenitor cells contributing to the ARC are housed within the hypothalamic ventricular zone (HVZ) where they receive cues to rapidly proliferate and remain undifferentiated (Ye et al., 2009). When progenitors are cued to differentiate, they will become post-mitotic and migrate out into the peripheral ARC where they will then begin expressing their unique neuropeptides (McClellan et al., 2008). A number of early patterning genes have been defined in the developing anterior diencephalon (Shimogori et al., 2010). Specifically, *Nkx2.1* is expressed broadly throughout the ventral border of the anterior hypothalamus corresponding with the tissue of the presumptive ARC (Nakamura et al., 2001). Following pattern specification, progenitors will begin differentiating into their respective mature cellular subtypes. It is becoming increasingly clear that each neuronal population is dependent on a distinct set of developmental factors. For instance, studies have shown the necessity for *Ngn3* in the development of both POMC and NPY neurons (Pelling et al., 2011). Similarly, *Gsh1* is critical for the formation of GHRH neurons (Li et al., 1996). Although few early markers of the developing ARC have been uncovered (Kimura et al., 1996; Wang and Lufkin, 2000; Cremona et al., 2004), the factors involved in the selection of a Kisspeptin fate remain largely unknown. Similarly, the lineage pathways shared between each neuronal subtype involved in maintaining energy balance and other homeostatic functions regulated by the ARC have yet to be uncovered.

The Notch signaling pathway is an evolutionarily conserved signaling pathway found throughout the developing embryo (Kortschak et al., 2001; Schroder and Gossler, 2002). In general, Notch signaling is a cell-to-cell dependent pathway by which a cell expressing one of the several Notch receptors receives a signal from a neighboring cell presenting a Notch ligand (Shimizu et al., 2000). Upon activation, the Notch intracellular domain (NICD) undergoes a series of cleavage events and will eventually translocate to the nucleus, where it will complex with a number of cofactors to initiate transcription (Struhl and Adachi, 1998). The most important factor is RBPJ-κ (*Drosophila* CBF1 (Olave et al., 1998)), which allows the activated complex to bind DNA and promote gene transcription (Nam et al., 2003). Notch signaling is implicated in two major steps in the progression of differentiation. First, as seen in the brain, liver, and other tissues, active Notch signaling is important in the decision of a progenitor cell to remain in a progenitor-like state or to exit the cell cycle and undergo differentiation (Qu et al., 2013; Bhat, 2014; Wang et al., 2014). However, Notch signaling is also important in selecting fates of cells which have begun differentiation. As seen in the immune system, active Notch signaling instructs intermediate hematopoietic stem cells to differentiate into immature T-lymphocytes and inhibits formation of B-lymphocytes (De Obaldia et al., 2013; Ayllon et al., 2015). Indeed, work from our lab and others has shown that Notch components as well as active downstream components of signaling are present in the presumptive hypothalamus (Casarosa et al., 1999; Aujla et al., 2013). We have previously shown that RBPJ-κ mediated Notch signaling is important for maintenance of ARC progenitors in the HVZ and plays a role in repressing the canonical pro-neural gene *Mash1* expressed within the ARC (Aujla et al., 2013). Conversely, loss of *Rbpj-κ* mediated Notch signaling leads to a premature burst of differentiation during early development, resulting in greater numbers of POMC and NPY neurons at e18.5.

The overall goal of this study is to provide further insight into how different neuronal subtypes of the ARC are specified from a common progenitor pool of the HVZ. Specifically, we hypothesized that the Notch signaling pathway would be involved in differentiation of Kisspeptin neurons of the ARC, and this neuronal subtype would arise from a common *Pomc* expressing intermediate progenitor cell. Utilizing both loss- and gain-of-function models, we have conditionally knocked out the RBPJ-κ component of the active Notch signaling complex (*Rbpj* cKO) or persistently expressed the constitutively active *Notch1* intracellular domain (ICD) (NICD tg) within *Nkx2.1* expressing cells in the developing ventral hypothalamus (Murtaugh et al., 2003; Tanigaki et al., 2004). In both models by which Notch signaling has been manipulated, a drastic reduction in Kisspeptin neurons occurs within the ARC. This suggests that persistent Notch signaling prevents neurogenesis irrespective of neuronal subtype; however, active Notch signaling is also important to instruct an immature neuron to adopt a Kisspeptin fate. This is not due to a differential timing of Kisspeptin neuron birth with respect to NPY neurons, and thus is not the result of progenitor depletion prior to Kisspeptin neurogenesis. Further, lineage tracing experiments have uncovered that a subset of Kisspeptin neurons of the ARC do indeed develop through a *Pomc* expressing intermediate progenitor, similar to NPY neurons. These findings taken together would suggest that *Rbpj-κ* dependent Notch signaling may be an important molecular switch to instruct early and/or intermediate progenitor cells to adopt the Kisspeptin identity.

2. Materials and methods

2.1. Mice

Rbpj-κ conditional knock-out (cKO) mice or *Notch1* intracellular domain constitutively active (NICD tg) mice were generated using established genetic mouse models. Rbpj^{tm1Hon} (*Rbpj-κ* floxed) mice provided by Dr. Tasuku Honjo (Kyoto University, Japan) (Tanigaki et al., 2004) or Gt(ROSA)26Sor$^{tm1(Notch1)Dam}$/J (Rosa^{Notch1ICD} floxed mice) (Murtaugh et al., 2003) purchased from Jackson Laboratories (Bar Harbor, ME, USA) were bred to C57BL/6J-Tg(Nkx2-1-cre)2Sand/J (*Nkx2.1*-cre) mice (Xu et al., 2008) purchased from Jackson Laboratories to generate *Rbpj* cKO and NICD tg mice, respectively. Lineage-tracing experiments to determine the origin of NPY and Kisspeptin neurons were performed by mating Tg(Pomc1-cre)16Lowl/J (*Pomc*-cre) mice purchased from Jackson Laboratories (Bar Harbor, ME, USA) (Balthasar et al., 2004) to B6.Cg-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J

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