Induction of Hypothalamic Neurons from Pluripotent Stem Cells

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Recently, various hypothalamic neurons have been successfully engineered from pluripotent stem cells, including mouse and human embryonic stem cells. Because pluripotent cells need to undergo stepwise changes during organogenesis, developmental analyses on the hypothalamus have been inevitable for numerous transcription factors that determine specification, survival, and migration during the formation of specific neurons. Hypothalamic progenitor cells arise from the retina and anterior neural fold homeobox (Rax)⁺ ventral part of the ventricular zone at embryonic day 10.5 (E10.5), and orthopedia (Otp) and steroidogenic factor-1 (SF-1) respectively appear in the dorsal and ventral regions at E13.5, which subsequently produce specific transcription factors required for the final maturation of hypothalamic neurons. In the pluripotent stem cells, rostrodorsal hypothalamic-like progenitors expressing retina and anterior neural fold homeobox are generated from floating aggregates in serum-free conditions with minimized exogenous patterning signaling. A certain population of the Rax⁺ progenitors generate Otp⁺ neuronal precursors, which subsequently develop into various dorsal and lateral hypothalamic neurons, including arginine vasopressin (AVP) and oxytocin neurons. Alternatively, early exposure to sonic hedgehog (Shh) induces differentiation markers including SF-1, specific for rostral–ventral hypothalamic-like precursors that eventually produce neuropeptide Y (NPY) and pro-opio-melanocortin (POMC). In conclusion, it is now possible to induce most types of hypothalamic neurons from pluripotent stem cells. Application of these cells would have advantages for studies on specification, migration, drug development, and regenerative medicine.

KEYWORDS: hypothalamus, embryonic stem cell, neuron, vasopressin

1. Introduction

The rostral hypothalamus is a relatively small, but critical, region wherein various nuclei are assembled for the orchestration of autonomic functions including energy homeostasis, feeding, temperature control, reproduction, growth, behavior, arousal, and fluid balance. Recent advances in research on the developmental markers required for specification, survival, and migration have helped to untangle the development of the large number of diverse neurons. Additionally, they have provided a roadmap for the differentiation process from tissue stem cells to mature neurons required for developmental engineering. In this minireview, we principally focus on the in vitro organogenesis of rostrodorsal neurons producing each of AVP, oxytocin, thyrotropin releasing hormone (TRH), corticotropin releasing hormone (CRH), or rostroventral neurons for neuropeptide Y (NPY) or proopiomelanocortin (POMC) from mouse or human pluripotent stem cells.

2. Development of the Rostral Hypothalamus

Recent studies have demonstrated that numerous transcription factors coordinating with one another to determine specification, survival, and migration are necessary for the formation of the hypothalamus, with individual neuronal subsets being guided by their own sets of factors [1].

At embryonic day 10.5 (E10.5) of mouse embryogenesis, the subventricular zone of the rostral forebrain is composed of two regions separated along the dorsoventral axis, comprising the telencephalon (brain factor-1:Bf1⁺) on the dorsal side, and the nontelencephalic (Bf1⁻) area on the ventral side that includes two major structures, the hypothalamus and the neural retina (Fig. 1A,B) [2]. A transcription factor, retina and anterior neural fold homeobox (Rax) is specifically expressed in the rostral hypothalamic neuroepithelium and retinal neuroepithelium [3]. The Rax⁺ hypothalamic neuroepithelium is different from the embryonic neural retina in that it expresses both Nestin and sex-determining region Y-box 1 (Sox1) [4]. The hypothalamic neuroepithelium is divided by the Nkx homeobox protein 2.2 (Nkx2.2)-positive region into two parts, comprising the rostrodorsal part that expresses relatively lower amounts of Rax and simultaneously expresses paired box protein 6 (Pax6) (Fig. 1C) and the rostroventral part that is Rax-rich and coexpresses Nkx2.1. The middle area expresses sonic hedgehog (Shh), a patterning signal responsible for the dorsoventral axis (Fig. 1D). The Pax6⁺ cells do not express Nkx2.1, and vice versa.

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At E13.5, a transcription factor, orthopedia (Otp), appears in the rostrodorsal part, and drives expression for the proliferation of progenitor cells destined to become the paraventricular nucleus (PVN), supraoptic nucleus (SON), and anterior periventricular nucleus (aPV) (Fig. 2). AVP or oxytocin neurons [1] in the PVN and SON are derived from Otp⁺/Brain2⁺ (Brn2⁺) precursors, and SON neurons are known to originate and migrate radially from the Otp⁺ subventricular zone. Otp-null mice do not form these nuclei, and do not express AVP, oxytocin, CRH, TRH, or somatostatin [5].

The rostroventral part develops two major nuclei that control energy metabolism, namely the arcuate nucleus (ARC) and ventromedial nucleus (VMN). The progenitor cells for most of these neurons [POMC, NPY/agouti-related peptide (AGRP), growth hormone releasing hormone (GHRH), dopamine, kisspeptin] are dependent on a helix-loop-helix transcription factor, achaete-scute complex 1 (ASCL1) [1]. ASCL1 is required for steroidgenic factor-1 (SF-1), which demarcates postmitotic VMN and POMC neuron precursors. ASCL1 also controls genomic screen homeobox I (GSH1) and zinc finger transcription factor Ikaros, which are necessary for the GHRH lineage.

In the mouse embryo, Shh is expressed in a subregion of the rostroventral hypothalamus (Fig. 1D). The gradation of Shh determines the dorsoventral axis in the rostral hypothalamus. Treatment with Shh increases SF-1⁺ neurons and decreases Otp⁺ neurons, and vice versa [2].

3. Neurogenesis from Pluripotent Stem Cells

With the aim of inducing various types of neural tissues from embryonic stem cells (ESCs), a culture method known as serum-free embryoid body-like aggregate quickly (SFEBq) is widely utilized (Fig. 3) [6]. ESCs are dissociated, and cultured as floating aggregates in 96-well plates with reduced cell-plate adhesion in chemically-defined, serum-free,
growth factor-free medium (gfCDM) that does not contain Wnt, Nodal, fibroblast growth factors (FGFs), bone morphogenetic proteins (BMP) (gfCDM). ESCs reaggregate quickly (within a few hours) and form uniformly sized masses. On day 3, expression of the earliest neuroectodermal marker Sox1 is first detected in the superficial zone of the SFEBq aggregates. The cells at this stage have pluripotency for various neuronal tissues. In the presence of Wnt antagonist dickkopf-related protein 1 (Dkk1), SFEBq induces efficient generation of cells expressing the telencephalic marker Bf1. Wnt3a treatment during the late culture period increases the pallial telencephalic population, while Shh promotes subpallial differentiation [7]. In a 3D culture matrix with Dkk1 and Nodal antagonist Lefty A, the retinal epithelium is generated [8]. Treatment with insulin and FGF2 induces cerebellar progenitors expressing a homeobox protein engrailed-2 (En2) [9].

4. Hypothalamic Induction from Mouse Embryonic Stem Cells

4.1 Removal of growth factors

For organogenesis of the rostral hypothalamus, Wataya et al. [2] identified a pivotal modification of gfCDM, the culture medium for the initial stage. Specifically, they removed insulin, which is widely used for neuronal culture. Exogenous treatment with insulin suppresses expressions of Rax and a paired box protein Pax6, which are necessary for the rostral hypothalamus. When mouse embryonic stem cells (mESCs) are cultured in insulin-free gfCDM, SFEBq aggregates express embryonic rostral hypothalamic markers including Rax, Nestin, and Sox1 at days 5–7 after induction (Fig. 4A). This profile of neuronal markers is different from that in the fetal retina, which is Rax⁺, but Nestin⁻ and Sox⁻ [2].
4.2 Purification of hypothalamic progenitor cells

Another point for rostral hypothalamic induction is the purification of hypothalamic progenitors from SFEBq aggregates at day 7 [2]. This is because octamer-binding transcription factor3/4\(^+\) (Oct3/4\(^+\)) or Nanog\(^+\) undifferentiated cells and progenitors for other tissues can remain, meaning that Rax\(^+\) cells need to be isolated for further neurogenesis. The isolated cells are reaggregated using SFEBq, and cultured until day 13. Without Shh, large numbers of cells express Otp (35% of total cells; Fig. 4B). Otp\(^+\) cells coexpress Brn2, which is correlated with the profile of the early rostroventral hypothalamus. AVP-producing neurons are found after day 18 of culture under either aggregation (Fig. 4C) or dissociation (Fig. 4D, E) conditions. These regenerated neurons are functionally compatible with vasopressinergic neurons in the SON or PVN, as they secrete AVP in response to depolarizing or hyperosmotic stimuli (Fig. 4F).

Shh treatment of SFEBq-gfCDM cultures from day 4 significantly increases SF-1\(^+\) cells and reduces Otp\(^+\) cell as investigated in the early rostroventral hypothalamus [2]. In the presence of exogenous Shh, Rax\(^+\) purified cells differentiate into VMN-like neurons that express SF-1 and glucose transporter 2 (GLUT2). Under the same culture conditions, other types of neurons in the rostroventral hypothalamus including NPY (Fig. 5E) or POMC (Fig. 5F) neurons are produced.

In human ESCs (hESCs), however, purification is not necessary for rostroventral hypothalamic induction [10]. Early activation of Shh signaling and SMAD inhibition (days 1–8) followed by inhibition of Notch signaling (days 9–12) induces hESCs to express Nkx-2.1, a rostroventral hypothalamic marker. Upon further treatment with brain-derived neurotrophic factor (BDNF) and Notch inhibitor, these cells express ASCL1, nescent helix loop helix 2 (NHLH2), or SF-1, which are required for differentiation and specification of hypothalamic neurons. After day 24, NPY/AGRP, POMC/cocaine- and amphetamine-regulated transcript (CART), somatostatin, and DA neurons appear in the culture. These studies illustrate that hESCs and mESCs share key molecules for development of the rostral hypothalamus. We cannot explain why hESCs do not require a purification process, but it may arise from their nature, given that they are less adhesive and require a longer time for maturation.
Recent progress in understanding transcription factors has clarified the cellular ontogenesis for the differentiation and specification of hypothalamic neuronal subtypes. By tracing the process \textit{in vitro}, regenerative engineering techniques have been successful in organogenesis of the hypothalamus from mammalian pluripotent stem cells. These systems will solve many questions regarding the developmental biology of the hypothalamus, including how terminal specification is determined, how migration occurs, and how axons are guided. Moreover, the \textit{in vitro} hypothalamic systems will be

Fig. 4. Ontogenesis of AVP neurons from mouse ESCs. (A) At day 7, the outer layer of an SFEBq aggregate expresses Rax. (B) At day 13, Opt$^+$ cells appear. (C) An AVP$^+$ neuron in membrane culture at day 28. Immunohistochemical staining for copeptin, an AVP marker, is shown. (D, E) An AVP neuron in dissociation culture at day 24 is shown (D), and some AVP neurons coexpress glutamic acid decarboxylase (GAD65) (E). Membrane cultures secrete AVP in response to 100 mM KCl, 230 mM NaCl, or 100 mM mannitol. For abbreviations, see text.

Fig. 5. Mediobasal hypothalamic neurons from mESCs. (A, B) NPY$^+$ neurons (A) and POMC$^+$ neurons (B) in dissociation culture at day 25 are shown. 4′,6-diamidino-2-phenylindole (DAPI) was used as a nuclear staining dye.

5. Conclusions

Recent progress in understanding transcription factors has clarified the cellular ontogenesis for the differentiation and specification of hypothalamic neuronal subtypes. By tracing the process \textit{in vitro}, regenerative engineering techniques have been successful in organogenesis of the hypothalamus from mammalian pluripotent stem cells. These systems will solve many questions regarding the developmental biology of the hypothalamus, including how terminal specification is determined, how migration occurs, and how axons are guided. Moreover, the \textit{in vitro} hypothalamic systems will be
applied for drug discovery in important areas such as energy homeostasis, temperature control, hormonal regulation, arousal, and circadian rhythms. The applications for regenerative medicine need to be considered with the aim of targeting hypothalamic dysfunctions arising from injuries, brain tumors, inflammatory diseases, and even genetic defects.

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