Cell-Based Models to Study GnRH Neuron Physiology

Abstract

The chance to produce *in vitro* cultures of neuronal cells has been fundamental for neurobiological studies. The advancing of our understanding on the physiology of the nervous system has been strongly supported by the *in vitro* cultures of neuronal cells, as both primary cultures and oncogene-mediated immortalized cells.

Among the hypothalamic neuroendocrine neurons, gonadotropin-releasing hormone (GnRH) expressing neurons represent a unique class; they are generated outside the brain, in the olfactory placode, and during embryonic life they move by tangential neurophilic migration, along terminal and vomeronasal nerves, to the septal-hypothalamic region. At this level GnRH neurons undergo terminal differentiation and axonal elongation to make contacts with the pituitary portal vessels in which they start releasing the decapeptide GnRH in a pulsatile fashion, to modulate the function of the reproductive axis.

However, the investigation of GnRH neurons has been hindered by their low abundance (800-1200) and their peculiar anatomical distribution. The study of these neurons has been forwarded since '90 by the availability of cell lines of immortalized mouse GnRH-expressing neurons (GT1 and GN cells lines); more later, other cell lines of GnRH-releasing neurons were established from human fetal olfactory neuroblasts and rat adult hypothalamic neurons under conditional immortalization. Later, different *in vitro* models for the study of GnRH neurons have been described; they include organotypic cultures of olfactory placode or hypothalamic tissue and primary cultures enriched in GnRH neurons. More recently, an new *in vitro* model of human GnRH neurons has been obtained from primary human fetal hypothalamic cell cultures. A new impulse to the study of these peculiar neurons in physiological, as well as in pathological conditions, will come from the already promising development of GnRH-secreting neurons from neuronal stem cells and induced pluripotent stem cells (iPSCs).

Keywords: Gonadotropin releasing hormone; Physiology; Cell-based model; Stem cells; Rodents, Human

Mini Review

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Abbreviations: SV40: Symian Virus 40; Tet-On: Tetracycline Inducible Expression Systems; HIV: Human Immunodeficiency Virus; SMAD: Small Mother Against Decapentaplegic proteins; FGF8: Fibroblast Growth Factor 8; Notch: Neurogenic Locus Notch Homolog Protein; BMP4: Bone Morphogenetic Protein 4

Introduction

The study of the Gonadotropin Releasing Hormone (GnRH)-secreting neurons in animal models is generally hindered by their peculiar development and anatomical distribution. Several subpopulations of GnRH neurons have been described; in addition, they born from the embryonic olfactory region (olfactory placode), migrate during a developmental narrow window of time (from embryonic day 11 to 18, in rodents and about in six weeks in human) and the GnRH neurons involved in the reproductive functions are very limited in number (from 800 in mouse to more than 2000 in human) [1,2] and scattered into the septo/hypothalamic region in adulthood.

The study of the factors that are directly involved in the mechanisms controlling the development and function of GnRH neurons requires the possibility to work in well-defined experimental conditions in which one can dissect out the specific actions of the several factors investigated.

Discussion

Rodent derived models

GnRH neuron immortalization: Actually, the study of GnRH neurons in the last decades has been facilitated by the development of cell models of isolated GnRH- secreting neurons.

We're more than 25 years from the establishment by Pamela Mellon and Sally Radovick groups of the first cell lines of immortalized mouse GnRH neurons. The different cell lines, named GT1 cells (which include GT1-1, -3 and -7 subclones) [3] and GN cells (with GN10, GN11 and NLT subclones) [4] have been obtained by genetically targeted tumorigenesis of GnRH neurons in mice using, respectively, the rat and human 5' regulatory region of the GnRH gene to control the expression of the SV40 large T-antigen oncogene (Figure 1).

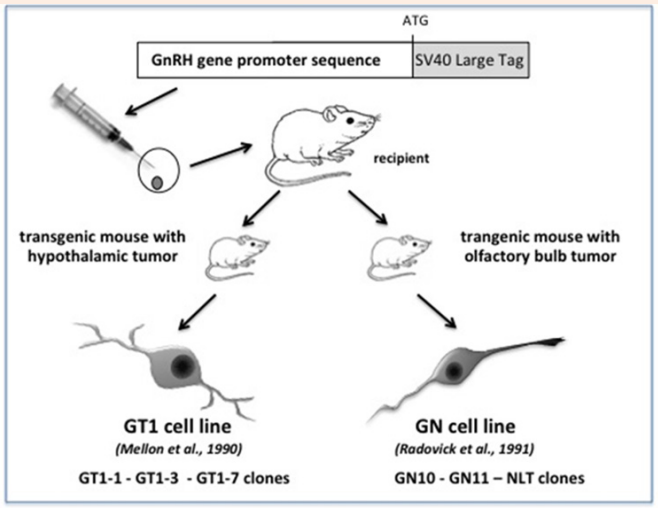

Figure 1: Schematic representation of the development of GT1 and GN cell lines.

Biochemical and immunological studies have shown that both cell lines express neuronal markers, and retain the biological features of GnRH-secreting neurons.

Later, we demonstrated for the first time that GN11 cells show a strong responses *in vitro* to several chemo-attractants with migratory characteristics and estimated migration speed (10 μm/h) very similar to that of brain neurons [5-8]; on the contrary, GT1-7 cells do not show significant migratory activity, unless under specific conditions [9].

These evidences are suggestive of GT1-7 and GN11 neurons as representative of differentiated post-migratory and immature migratory GnRH neurons, respectively [5,6]. Moreover, the use of these models in more than 370 published studies (source PubMed) and the finding of a number of similarities with normal GnRH neurons "*in vivo*" [5,10-12] confirm that immortalized GnRH neurons could be considered an adequate model to study the biology of GnRH-secreting neurons in isolated conditions.

Thanks to the *in vitro* migratory activity of GN11 cells, we have provided the first experimental evidence that the product of ANOS1 gene (anosmin 1) could exert a direct stimulatory action on the migration of GnRH neurons [7]. ANOS1 is the first gene identified as causative of the Kallmann's Syndrome (KS), a genetic hypogonadotropic hypogonadism due to GnRH deficiency [13]. GN11 cells have been also successfully used to investigate, at the cell level, or to confirm the role of several factors found to affect the migration of wild type mouse GnRH neurons [14-20].

GT1 cell clones have been widely used to study the electrophysiological characteristics of GnRH neurons that lead to the pulsatile release of the decapeptide [21,22] as well as the transcriptional control of GnRH gene and the effects of several regulators of GnRH secretion [23-26].

GT1 and GN derived neurons have been also used to evaluate many physiological mechanisms involving GnRH [27]. Recently, GnRH neurons have been proposed as a target of energy sensors and immortalized neurons offer a useful tool to study the effects of altered energy/metabolic state on GnRH expression or neuronal migration. Both GT1-7 and GN11 express insulin receptors, that mediate glucose uptake and phosphorylation of Akt through IP3kinase activation. GT1-7 cells has been also used to develop an *in vitro* model of neuronal insulin resistance [28]. The energy sensor adenosine monophosphate-activated protein kinase (AMPK) is also expressed in both GT1-7 and GN11 and it is involved in the regulation of a Glucose Excite-type glucose-sensing behavior and cell migration, respectively [29,30].

On the whole, these cell lines represent ideal models for testing the role of factors involved in GnRH neuron survival, differentiation and migration in controlled *in vitro* conditions, offering the unique opportunity to induce genetic modifications of isolated GnRH neurons as well as to expose them to altered metabolic environments that may be causative of several diseases.

In order to ascertain the potential direct effect of insulin, another cell line of rat GnRH neurons has been described by Salvi et al. [31]. These neurons (Gnv-3) have been selected from twelve different clonal cell lines that were derived from primary cultures of adult (10-12 week-old female) rat hypothalamic neurons by conditional immortalization; these cells retain many of their mature phenotypic characteristics of mature GnRH neurons. In contrast to GT1 cells, the GnV cells are under a conditional immortalization process obtained using a Tet-On system (55) transfected in primary neurons by lentiviral vectors derived from HIV; these vectors are able to transduce non dividing, post mitotic neurons, and have been successfully used in other studies [31,32].

Immortalized GnRH neurons offer many advantages in term of stability, repeatability, easiness of culture and gene transfection that make them useful for complex cell biology studies.

Primary tissue and cell cultures: To circumvent the possible problems linked to the oncogene-derived immortalization of the cells, slice explant culture method was also developed to study adult GnRH neurons [33]. In particular, organotypic cultures of explants of the hypothalamus or the preoptic area to study adult GnRH neurons have yielded interesting results on neuronal function and decapeptide secretion [34,35]. GnRH neurons in tissue explants retain features of their *in vivo* counterparts and maintain many physiological connections with neighboring cells (neurons or glial cells) but they were not so efficient to directly test isolated GnRH neurons, mainly because of their post-hoc identification [36].

The embryonic expression of GnRH gene allowed the setup of cultures of olfactory placode explants [37-42]. This model was developed for mouse, rat, sheep and Rhesus monkey and it presents several advantages since GnRH neurons in the placode have not been in contact with hypothalamic neurons or glial cells and therefore they have not still established a physiological state of secretion. However, this type of culture represents an interesting model limited for studying the mechanisms underlying the onset and the control of pulsatility in GnRH neurons.

The difficulties to work on isolated native GnRH neurons have been overcame by efficient procedures of cell isolation and cultures.

Several studies have described the isolation of primary cultures of hypothalamic and of nasal placodes derived neurons [41,43,44].

Primary culture of GnRH neurons derived from embryonic olfactory placode has been obtained from different animal species (mouse, rat, sheep and monkey) and used to study several mechanisms implied in GnRH neuron development and function [37,38,40,45,46]. In these cultures olfactory neurons and olfactory ensheathing cells are maintained and therefore GnRH neurons result still mixed with other cell populations.

To facilitate the study of GnRH neurons in tissue explants, transgenic mice with jellyfish reporter, green fluorescent protein (GFP), targeted to GnRH neurons (GFP-GnRH mice) have been developed [47]. Primary cultures of GnRH neurons have also obtained by dispersion of fetal olfactory tissue derived from of GFP-GnRH mice [48,49]. The presence of GnRH neurons expressing GFP in tissue explant or mixed cell cultures allows to identify living GnRH neurons in tissue slice preparations before and during the experiments; this offers significant experimental advantages for biochemical or electrophysiological experiments [50,51].

Human-derived models

The complete inaccessibility of human GnRH neurons also in *ex vivo* studies rose the need of a specific cell-based model.

In 1995 it has been reported the isolation, characterization, and cloning of neuroblast cell lines obtained from olfactory epithelium of 8- to 12-week-old human fetuses that showed typical neuronal and olfactory properties and were able to respond to odors [52]; later it has been demonstrated that one such clone (FNC-B4) produced GnRH, it was responsive to sex steroids and sensitive to odorants in terms of GnRH production [53]. FNC-B4 showed migratory activity *in vitro* [54] and have been used in several protocols of investigation on human GnRH neuron functions [55-57]. Moreover, these cells express several genes relevant for GnRH neuron function as kisspeptin and its receptor, sex steroid, leptin and FGF receptors [56,58].

More recently, another *in vitro* model of GnRH neurons of human origin was recently obtained from primary human fetal hypothalamic cell cultures isolated from brain of three 12 week-old fetuses (hfHypo cells) [59]. These neurons were found to be responsive to kisspeptin and were successfully used to investigate the effects of pro inflammatory cytokines on their biological properties, as previously observed by *in vivo* experiments. The hf Hypo cells represent a novel tool for *in vitro* investigations on the human GnRH neuron biology and its involvement in hypothalamic inflammation, a process that has been implicated in programming systemic aging [60].

Neuronal stem cells and induced pluripotent stem cells (iPSCs)-derived cell models: The future improvement of the study of GnRH neuron pathophysiology might stem from the establishment of *in vitro* models derived from neuronal multipotent or pluripotent precursor cells. Salvi et al. [31] isolated NSCs from rat embryonic (E18) and adult hypothalamus; these cells grow in suspension as neurospheres and once differentiated they exhibit a GnRH-like neuronal subtype [61], possibly indicating that they represent a GnRH-committed population of olfactory placode-derived precursors.

However, isolation and characterization of a cell line of embryonic stem cells (AC1) from E14 mouse hypothalamic region (when GnRH neurons are still migrating from the olfactory region) failed to show GnRH expression also under high-efficiency neuronal differentiating conditions [8]. These results suggest a clear difficulty to isolate GnRH-committed NSC to be easily used for *in vitro* studies.

Recently, a three-step protocol to differentiate human pluripotent stem cells (hPSCs) into GnRH-secreting neurons has been reported [62]. The hPSCs were first differentiated to anterior neural progenitor cells (NPCs) by dual SMAD inhibition, then exposed to FGF8, a factor involved in GnRH neuron ontogeny, and finally matured to neurons by Notch inhibitor. These cells have been shown to express the GnRH gene and to release the GnRH decapeptide into the culture medium. The protocol was found to be reproducible both in human Embryonic Stem Cells (ESC) and iPScs.

Finally, it has been reported the efficient generation of GnRH-secreting neurons by directed differentiation of human ESC with a an alternative protocol [63]. These neurons have been generated by a long-term exposure of ESC to BMP4 inhibition to induce intermediate NPCs, which have been then terminally specificated with FGF8 and Notch inhibitor. The resulting neurons express and release GnRH, display a neuroendocrine gene expression pattern and present spontaneous calcium transients that can be stimulated by kisspeptin. Of interest, similar GnRH cells have been obtained from induced-Pluripotent Stem Cells derived by a KS patient.

Once better characterized to be responsive to known regulatory signals, these *in vitro* generated GnRH expressing cells will open new perspectives in the investigation of the molecular mechanisms underlying the development and function of human GnRH neurons.

Conclusion

The development of cell-based models of GnRH neurons represents an ideal tool for testing the role of the factors involved in GnRH neuron survival, differentiation and migration in controlled *in vitro* conditions and offers the unique opportunity to induce genetic modifications as well as to test the effects of altered metabolic environments.

The study of the isolated GnRH neurons is of true neurobiological and neuroendocrinological interest. It has potential implications not only for the investigation of the molecular mechanisms leading to the development of reproductive functions, as well as their related diseases (such as central hypogonadotropic hypogonadisms), but also for the variable, concomitant neurological defects occurring in addition to the overt infertility and for the screening of possible therapeutic molecules. Not least, the study of the molecular cues that affect the development and functions of GnRH neurons may also provide novel insights on common biochemical events controlling the physiology of other neuronal populations.

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Conflict of Interest

The author certifies that they have NO affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript.

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