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Unique Estrogenic Mechanisms for Unique Gonadotropin-Releasing Hormone Neurons?

The history of studies of mechanisms of estrogen action in the brain is filled with at least two periods of intense dogmatic beliefs. These include thinking that a single type of cytoplasmic, unliganded estrogen receptor (ER) bound ligand and moved to the nucleus after activation (1), to thinking that all ERs were located in cell nuclei even in the unoccupied state (2). The dogma of cytoplasmic *vs.* nuclear distribution began to die in the early 1990s with the recognition of a more widespread intracellular distribution (3), and the single ER concept died later in the 1990s with the discovery of ER-β (4). The current state of affairs was well summarized recently (5, 6) as a situation in which a plethora of proteins localized from plasma membrane to nuclei has the potential to bind estrogens and initiate diverse signal transduction pathways.

A history of studies of mechanisms governing the functioning of GnRH neurons has also broken through several dogmatic stretches, including the once-held belief that GnRH neurons contain few if any hormone receptors, particularly ERs (7). Nevertheless, the importance of hormone modulation of GnRH physiology (whether direct or indirect) has long been recognized. Despite a long history of studying estrogen effects on the hypothalamic-pituitary-gonadal axis and GnRH neurons, investigations of hormone influences on GnRH neurons have remained limited by an 800-lb gorilla in the room; the considerably heterogeneous GnRH neuronal network consists of relatively few cells widely distributed throughout the forebrain, making single-cell investigations a prodigious challenge.

Significant progress on teasing out how GnRH neurons work can be traced first to the development of *in vitro* approaches to identifying GnRH neurons (*e.g.* Ref. 8), and then to the development of transgenic mice in which the GnRH promoter drives the expression of enhanced green fluorescent protein (9). These advances, together with others in molecular genetics and the use of immortalized GnRH neuronal cell lines, have allowed researchers to begin investigating hormone feedback to GnRH neurons with single cell resolution, notwithstanding challenges presented by their scattered topography. The march of progress now brings the ever-expanding field of ER signaling barreling down a track toward the field of GnRH neuronal function at the single cell level with ever increasing frequency. The crash, seemingly analogous to those produced in a particle accelerator, seems to throw out new "particles" with each collision.

A recent report in *Endocrinology* (10) used an explant model derived from primate olfactory placodes to describe rapid increases in intracellular Ca^{2+} oscillations mediated by estrogens signaling through a membrane/cytoplasmic ER. This is not the first report of Ca^{2+} oscillations in GnRH neurons as the authors point out; however, the new data point to the G protein-coupled receptor, GPR30, as the source of the signaling rather than ER-α or β. In the current issue of *Endocrinology*, two groups present further novel data on estrogenic influences on GnRH neuron physiology using brain slices and GnRH neurons identified by their expression of GFP (11) or a genetically encoded calcium indicator, pericam, under control of the GnRH promoter (12). Use of the calcium indicator revealed rapid effects of estradiol on calcium dynamics in murine GnRH neurons. Activation of intracellular calcium transients was observed approximately 15 min after treatment with 17-β-estradiol through a mechanism shown to be selective for intracellular ERα as compared with a membrane located ERα, ERβ, or GPR30. Interestingly, blocking action-potential dependent synaptic transmission with tetrodotoxin was not sufficient to abolish the ER-dependent transients, whereas tetrodotoxin treatment in the presence of a γ-aminobutyric acid-$(GABA)_A$ receptor antagonist completely inhibited stimulation of transients. Together, these experiments revealed a nonclassical estrogenic modulation of presynaptic γ-aminobutyric acid-ergic terminals that then impacts a subset of GnRH neurons. In a separate study, a novel combination of knockout and transgenic mice provided evidence that classical genomic ERα-based estrogen response element (ERE)-dependent signaling is necessary for the proper regulation of negative and positive estrogen feedback at the level of the GnRH neuron (11). A line of mice was bred that expressed a knock-in allele that selectively restores a modified ERα that can no longer bind to EREs on an ERα knockout background that also carries the transgene for GnRH promoter driven enhanced green fluorescent protein expression. Using these mice the authors were able to differentiate ERE-dependent and ERE-independent mechanisms of GnRH neuron activation. GnRH neuron firing rates were altered in brain slices from mice placed in situations of either positive or negative feedback in ERα knockout mice, or in ERα mice with selectively restored ERE-independent signaling. These data suggest that classical negative and positive feedback is dependent on genomic ERα interactions with ERE, although the cellular locus of estrogenic signaling was not determined. Because the manipulated ER was the α-form, the cellular locus is presumed to be other than GnRH neurons themselves. Together, these studies provide additional evidence that a variety of estrogenic mechanisms may influence GnRH neurons.

The authors of these papers (10–12) eloquently review past history in the field that is directly relevant to their results. In this commentary we take a step back and look at the emerg-

See articles p. 5328 and 5335.

Abbreviations: ER, Estrogen receptor; ERE, estrogen response element.

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ing results from the perspective of that 800-lb gorilla. This is important because at a superficial level, these results may seem to be contradictory (*e.g.* Why are rapid estrogenic effects mediated by both membrane and cytoplasmic ERs by both genomic and nongenomic mechanisms?). However, there are good reasons to believe that all these mechanisms might be part of the same fabric of the GnRH neuronal network, weaved in as separate estrogen-responsive subpopulations. Although GnRH neurons exhibit critical periodic network synchronization, it has long been evident that whether viewed from the perspective of subpopulations of GnRH neurons, or at the level of single GnRH neurons, there is astonishing heterogeneity. This is equally true whether one considers GnRH neurons from the perspective of development (*e.g.* Refs. 13 and 14) or adulthood (*e.g.* Refs. 15 and 16). Thus, it is important to view mechanistic studies as investigations into GnRH neurons on multiple levels, ranging from individual units to subpopulations and larger networks. From this perspective it might be expected that multiple estrogenic mechanisms regulate similar or different types of GnRH neurons.

Under certain circumstances the challenges inherent to studying complex situations such as estrogen signaling to a phenotypically heterogeneous population may be exacerbated by the increasing complexity of the methodology used. This theme appears to be exemplified by the extensive data emerging on the interactions between estrogens and GnRH neurons. As separate groups use novel genetic models and specialized reagents to produce compelling new data, the studies become more complicated (and sometimes impractical) to reproduce. To draw comparisons between data from different groups and reduce the results to a meaningful body of work, it is becoming more important that the studies be analyzed and interpreted in an appropriate context. What was the model species? Were the GnRH neurons derived from embryonic, prepubertal, or postpubertal animals? How were the neurons cultured: dissociated or in slices? Do the methods bias the selection of specific subsets of cells with unique characteristics, based on location, specialized promoter activity, or something else?

In regards to the studies appearing herein, as well as a number of other reports on GnRH physiology, the set of answers to each of the aforementioned questions is variable. It is not likely that investigators will reach strict agreement on model systems or approaches; thus, rather than analyzing studies relative to each other, the challenge that we face going forward is to deduce how any given experimental system relates to *in vivo* estrogen signaling to GnRH neurons. Each data set is informative about interactions between estrogens and GnRH neurons under specific experimental constraints, and can be informative if viewed from the proper perspective. To expand upon the works recently published, it will be important to delineate further how estrogenic signaling to GnRH neurons differs across developmental stages, hormone states, and species. It will also be interesting to see the degree to which distinct estrogen-responsive subpopulations exist within the GnRH neuronal network. As these questions are answered, it may become more apparent how evolution has weaved each estrogenic pathway into the complex GnRH neuronal network that is essential for the survival of all species.

There will likely be no simple model. Estrogens can affect GnRH neurons directly or indirectly, through classical ERα or ERβ mechanisms, or through other recently described membrane-associated ERs (5, 6). As evidence that a plethora of estrogenic mechanisms impinges upon GnRH neurons mounts, it may be that research on the interactions between estrogens and GnRH neurons will require a paradigm shift. Progress will require acknowledgment of diverse estrogen-GnRH neuron interactions and an understanding that these mechanisms might interact. It is not yet feasible to declare any one set of mechanisms as canonical for estrogenic signaling to GnRH neurons, and it seems unlikely that one will be found.

As molecular investigations of estrogen signaling collide with GnRH neuron physiology, how do we piece the "particles" together as old dogmas are shed? Multiple groups have advanced our understanding of potential estrogenic signaling pathways affecting GnRH neurons, yet the field still searches for a consensus view of estrogenic influences. Future success might lie in our ability to collaborate, to find ways to exploit the advantages that separate groups have, to work together to design experiments that we can reach consensus conclusions from. Competition can be a power-driving force for scientific advancement; however, for some compelling questions, maybe a few well-planned experiments to settle friendly wagers will prove to be a useful tool to move science forward.

Brandon C. Wadas and Stuart A. Tobet
Department of Biomedical Sciences
Colorado State University
Fort Collins, Colorado 80523

Acknowledgments

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Address all correspondence and requests for reprints to: Stuart Tobet, Colorado State University, Department of Biomedical Sciences, 1617 Campus Delivery, Fort Collins, Colorado 80523. E-mail: stuart.tobet@colostate.edu.

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