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Review

An integrative overview of the role of gonadotropin-inhibitory hormone in behavior: Applying Tinbergen's four questions

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ABSTRACT

The integration of various fields of investigation is of key importance to fully comprehending endocrine function. Here, I enact the theoretical framework of Nikolaas Tinbergen's four questions for understanding behavior to help bridge the wide gap that exists between our relatively reductionist molecular knowledge of a particular neurohormone, gonadotropin-inhibitory hormone (GnIH), and its place in animal behavior. Hypothalamic GnIH, upon its discovery in 2000, was so named because of its inhibitory effect on the release of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), from the pituitary. Because gonadotropins are necessary for reproduction, this finding stimulated questions about the functional significance of GnIH in reproduction and sexual behavior. After over a decade of research, invaluable knowledge has been gained regarding the mechanistic attributes of GnIH (mammalian homolog, RFamide-related peptide (RFRP)) in a variety of vertebrate species. However, many questions remain regarding the effect of the environment on GnIH and the subsequent effects of GnIH on behavior. I review the role of GnIH in shaping behavior using the framework of Tinbergen's four questions of mechanism, ontogeny, function and phylogeny. The studies I review were conducted in various species of mammals, birds, and in one species of fish. Because GnIH can play a role in mediating behaviors such as those important for reproduction, sociality, feeding, and the stress response in a variety of species, an integrative approach to the study of GnIH will help provide a multipronged schema for answering questions of GnIH function. By using the framework highlighted by Tinbergen's four questions, we will deepen and enhance our knowledge of the role of hormones in behavior from the point of view of the mechanisms involved.

1. Introduction

Dutch ethologist and Nobel Prize winner Nikolaas Tinbergen (1907–1988) highlighted four major questions that have become a cornerstone template for understanding animal behavior (Tinbergen, 1963). These questions are as follows: (1) What is the mechanism that elicits the behavior? (2) How does the behavior change within an individual during development? (3) How does the behavior affect the organism's chances of survival and reproduction? and (4) How did this behavior evolve? The first two questions of mechanism and ontogeny are generally thought to address the "proximate mechanisms" of behavior, while the latter questions of adaptation and evolution address the "ultimate bases" for behavior (Mayr, 1961). The application of these questions is intended to yield a true integrative understanding of the animal behavior in question. Fifty years later, these questions are still considered a cornerstone for gaining a comprehensive, multifaceted understanding of behavior (Ophir, 2011; Baterson and Laland, 2013; Barrett et al., 2013).

An insightful review by MacDougall-Shackleton et al. (2013) expands on the benefits of using integrative approaches and different levels of analysis to understand behavior. Specifically, consideration of the ultimate function of a behavior can aid in exploring the mechanisms driving it (MacDougall-Shackleton et al., 2013). For the sake of the present review, we will stray with Tinbergen's looking glass from behavioral phenotypes to hormonal phenotypes, as these four questions are intended to be applicable to any phenotype, and a hormone and its functions can also be viewed as phenotypic characteristics. With this in mind, I offer a novel way of examining the role of a specific neuropeptide, gonadotropin-inhibitory hormone (GnIH), in mediating certain aspects of reproduction and feeding behaviors. Although GnIH was discovered in 2000 (Tsutsui et al.), very little is understood about how changes in GnIH manifest behaviorally and how the environment,

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in turn, affects GnIH. Without a greater understanding for how GnIH functions in and responds to the external world, we will never completely grasp its biology.

Using the Tinbergian framework, I review what is known about the role of GnIH (and its mammalian homolog, RFamide-related peptide (RFRP)) in behavior, both proximately and ultimately, and highlight what questions remain unanswered. The goal of this review is to help inspire and direct future research aimed at uncovering the roles of GnIH in behavior. Additionally, I hope to create theoretical infrastructure to help bridge the wide gap that exists between our molecular knowledge of GnIH and its place in evolutionary biology and animal behavior.

2. Proximate views: mechanistic and ontogenetic perspectives

2.1. Mechanism. How does GnIH work?

Over a decade of research on GnIH has been instrumental in revealing its mechanistic properties. GnIH can inhibit reproductive physiology in a number of ways (but see Koda et al. (2002), Ukena et al. (2003a), Amano et al. (2006), Revel et al. (2008) and Moussavi et al. (2013)). Because numerous reviews have provided excellent explanations of and extensive resources pertaining to the mechanistic properties of GnIH, I will offer only a brief overview of its physiological function. These previous reviews have helped to elucidate many of the regulatory mechanisms of GnIH synthesis and release, the role of GnIH receptor in GnIH-induced cell signaling, and the comparative physiology, seasonality, neuroanatomy, and modulation of GnIH in the brain, pituitary and gonads in a variety of taxa. For more in-depth information, please see Bentley et al. (2006a,b,c, 2007, 2009a,b, 2010), Kriegsfeld (2006), Tsutsui (2006, 2010), Tsutsui et al. (2006, 2007a,b, 2009, 2010a,b,c, 2012, 2013), Greives et al. (2008), Tsutsui and Bentley (2008), Ubuka et al. (2008b, 2012c, 2013), Tsutsui and Osugi (2009), Smith and Clarke (2010), Ubuka and Bentley (2011), Parhar et al. (2012) and Chowdhury et al. (2013), Tsutsui and Ubuka (2014); Table 1.

The discovery of GnIH fundamentally changed the accepted understanding of the regulation of reproduction. Vertebrate reproduction is regulated by the hypothalamic neurohormone gonadotropin-releasing hormone (GnRH). GnRH can exist in several forms, depending on the species. GnRH is released from neurons in the preoptic area of the hypothalamus to the median eminence, causing the pituitary gland to secrete the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the bloodstream. LH travels to the gonads, where it stimulates the production of reproductive steroids such as androgens and estrogens, whereas FSH guides gamete production. The sex steroids provide feedback to the brain and pituitary, creating a regulatory feedback system necessary for reproduction and many associated behaviors. The framework commonly used to compartmentalize and discuss such physiological function is referred to as the hypothalamic–pituitary–gonadal (HPG) axis, or more colloquially as the Reproductive Axis (Fig. 1). In teleost fishes, dopamine has been known as a gonadotropin-inhibiting hormone for some time (Chang and Peter, 1983; Van Der Kraak, 2009; Zohar et al., 2010). However, in mammals and birds, neural inhibition of gonadotropins was thought to be solely the result of increased feedback into the brain from the pituitary and gonads. The discovery of GnIH and its active inhibitory effects on the Reproductive Axis altered that dogma.

GnIH was first isolated from quail (*Coturnix japonica*) brains and was found to dose-dependently inhibit the release of LH from cultured quail pituitaries (Tsutsui et al., 2000). Because of this, it was named gonadotropin inhibitory hormone. We now know that GnIH can inhibit gonadotropin release *in vivo* in birds (e.g., chicken (Ciccone et al., 2004), quail (Ubuka et al., 2006) and white crowned sparrows (*Zonotrichia leucophrys gambelii*) (Osugi et al., 2004)) and mammals (e.g., hamsters (*Mesocricetus auratus*) and mice (*Mus musculus*) (Kriegsfeld et al., 2006), rats (*Rattus norvegicus*) (Kriegsfeld et al., 2006; Johnson et al., 2007) and sheep (Sari et al., 2009). Intraperitoneal administration of GnIH also inhibits gonadotropin release in goldfish (Zhanga et al., 2010).

GnIH-producing neurons are located in the paraventricular nucleus (PVN) of birds. Its mammalian homolog, RFRP, is found in the dorsomedial hypothalamus (DMH) of rodents (Kriegsfeld et al., 2006). GnIH fibers are widespread in the diencephalic and mesencephalic regions of birds and mammals (e.g., quail: Ukena et al., 2003b; European starlings: Ubuka et al., 2008a; white-crowned sparrows: Ubuka et al., 2012b; rats: Johnson et al., 2007; Siberian hamsters: Ubuka et al., 2012a; monkey: Ubuka et al., 2009b). McGuire et al. (2013) have reported GnIH presence in zebra finch hippocampal fibers. These data and more (review: Ubuka et al., 2012a,b,c) suggest a role for GnIH beyond only gonadotropin regulation, though our current understanding of that role is very limited.

Central administration of GnIH can decrease copulation solicitations in birds (Bentley et al., 2006a) and sexual behaviors in rodents (Johnson et al., 2007; Piekarski et al., 2013). GnIH-containing cellular projections appear to make direct contact with gonadotropin-releasing hormone neurons in both birds and mammals (Bentley et al., 2003, 2006b; Kriegsfeld, 2006; Ubuka et al., 2008a) providing the architecture for direct communication to, and inhibition of, the HPG axis and associated sexual and reproductive behaviors. These studies (also see previously listed reviews) demonstrate that GnIH can directly inhibit the HPG axis by decreasing the activity of GnRH neurons and reducing the synthesis and release of the gonadotropin LH and in some cases FSH from the pituitary gland, and testosterone release from the gonads.

The majority of knowledge we have gained since the discovery of GnIH has centered on its peptide structure and mechanism of action. This is exciting because when exploring the intricacies driving behaviors, proximate mechanisms are usually the least elucidated (Tinbergen, 1963; Baterson and Laland, 2013). However, in this case, we know very little concerning the role of GnIH during ontogeny, its impact on survival and reproduction, and its evolutionary history. While more intricate knowledge of its internal mechanistic properties is important for understanding of GnIH, there exists a need to explore these latter aspects as well. As Tinbergen (1963) urged and Baterson and Laland (2013) stressed, it is the *integration* of knowledge gained from the four questions that will yield a deeper understanding of the characteristic of interest.

2.2. Ontogeny. How does the role of GnIH change during development?

The study of a trait over the course of an individual's development can help clarify its purpose. Classic experiments on early critical periods in development for filial imprinting and song and language learning have demonstrated that both nature (innate priming of the system) and nurture (experience and environment) can affect behavior at different stages during an individual's life. Very little is known about the role of GnIH driving ontogenetic transitions or shifts in GnIH function across ontogeny. Is its action solidified at birth or can environmental perturbations change the way its function manifests behaviorally?

Hypothalamic GnIH precursor mRNA, mature peptide and fibers are expressed in quail embryos on embryonic day 10 (E10) and show a significant increase in abundance right before hatch on E17 (Ubuka et al., 2003). GnIH content decreases post-hatching but then progressively increases into adulthood, at which time GnIH fibers extend to the external layer of the median eminence.

Table 1
A collection of reviews and studies of GnIH as categorized by what aspect(s) of Tinbergen's four questions they address: mechanism (How does GnIH work?), ontogeny (How does the role of GnIH change during development?), function (How does GnIH enhance survival and reproduction?), and phylogeny (How did GnIH evolve?).

Proximate	Sub-category	Review paper or species	Report	Citation
Mechanism:		Review paper	Driving reproduction: RFamide peptides	Kriegsfeld (2006)
		Review paper	GnIH: Discovery, progress and perspective	Tsutsui (2006)
		Review paper	Mode of action and functional significance of avian GnIH: A review.	Tsutsui et al. (2006)
		Review paper	Interactions of GnRH and GnIH in birds and mammals	Bentley et al. (2006b)
		Review paper	GnIH in seasonally breeding songbirds: Form and function	Bentley et al. (2006c)
		Review paper	GnIH in seasonally-breeding songbirds: neuroanatomy and functional biology	Bentley et al. (2007)
		Review paper	Discovery of GnIH in a domesticated bird, its mode of action and functional significance	Tsutsui et al. (2007b)
		Review paper	The general and comparative biology of GnIH	Tsutsui et al. (2007a)
		Review paper	Recent advances in reproductive neuroendocrinology: a role for RFamide peptides in seasonal reproduction?	Grieves et al. (2008)
		Review paper	GnIH: biosynthesis, mode of action and functional significance in birds	Tsutsui and Bentley (2008)
		Review paper	The control of reproductive physiology and behavior by GnIH	Ubuka et al. (2008a,b)
		Review paper	GnIH: A multifunctional neuropeptide	Bentley et al. (2009a)
		Review paper	A new key neurohormone controlling reproduction, GnIH: Biosynthesis, mode of action and functional significance	Tsutsui (2009)
		Review paper	A new key neurohormone controlling reproduction,GnIH in birds: Discovery, progress and Prospects	Tsutsui et al. (2009)
		Review paper	Evolutionary origin and divergence of GnIH and its homologous peptides	Tsutsui and Osugi (2009)
		Review paper	Recent studies of GnIH in the mammalian hypothalamus, pituitary and gonads	Bentley et al. (2010)
		Review paper	GnIH function in mammals	Smith and Clarke (2010)
		Review paper	Phylogenetic aspects of GnIH and its homologs in vertebrates	Tsutsui (2010)
		Review paper	GnIH and its control of central and peripheral reproductive function	Tsutsui et al. (2010a)
		Review paper	Discovery and evolutionary history of GnIH and kisspeptin: New key neuropeptides controlling reproduction	Tsutsui et al. (2010b)
		Review paper	Discovery and functional significance of GnIH in vertebrates: From comparative to general	Tsutsui et al. (2010c)
		Review paper	Neuroendocrine control of reproduction in birds	Ubuka and Bentley (2011)
		Review paper	RFamide peptides as mediators in environmental control of GnRH neurons	Parhar et al. (2012)
		Review paper	GnIH: Discovery, progress and prospect	Tsutsui et al. (2012)
		Review paper	GnIH action in the brain and pituitary	Ubuka et al. (2012c)
		Review paper	Review: Melatonin stimulates the synthesis and release of GnIH in birds	Chowdhury et al. (2013)
		Review paper	Review: regulatory mechanisms of GnIH synthesis and release in photoperiodic animals	Tsutsui et al. (2013)
		Review paper	GnIH, GnIH receptor and cell signaling	Ubuka et al. (2013)
		Review paper	GnIH regulates reproduction and reproductive behavior	Tsutsui and Ubuka (2014)
Ontogeny:		quail	GnIH mRNA, peptide and fibers increase in chick embryos right before they hatch	Ubuka et al (2003)
		zebrafish	GnIH expression is detectable at all embryonic stages, hatching, early development. Expression patterns differes amoung various stages	Zhanga et al. (2010)
		male mice	GnIH expression in the testes increases from the prepubertal to the pubertal period	Anjum et al. (2012)
		female rats	GnIH expression varied during development, showing a decline during the transition into puberty	Maze et al. (2013)
Ultimate				
Function:	Stress	male and female house sparrows	GnIH peptide cell abundance changes in response to stress during the breeding season but not the non-breeding season	Calisi et al. (2008)
		male Sprague-Dawley rats	GnIH peptide cell abundance changes in response to stress, and adrenalectomy blocked this response. Glucocorticoid receptors co-express with GnIH-ir cells	Kirby et al. (2009)
		European starling	Glucocorticoid receptors co-express with GnIH-ir cells	Calisi et al. (2010)

(*continued on next page*)

Table 1 (*continued*)

Proximate	Sub-category	Review paper or species	Report	Citation
	Feeding	red junglefowl chicks	ICV administration of GnIH stimulates food intake	Tachibana et al. (2005)
		male Sprague-Dawley rats	ICV administration of GnIH stimulates food intake	Johnson et al. (2007)
		female Syrian hamsters	Food restriction increased double-labeled cells for IEG and GnIH ('activation'), but the number of GnIH cells decreased. Activation was associated with increase in food hording but not food ingestion	Klingerman et al. (2011)
		male and female sheep, mice, cynomolgus macaques	ICV administration of GnIH stimulates food intake without affecting energy expenditure or sexual behavior	Clarke et al. (2012)
	Reproductive behavior	female white crowned sparrows	ICV administration of GnIH increased copulation solicitation displays in response to male song, but it did not affect ambulatory behavior	Bentley et al. (2006)
		male Sprague-Dawley rats	ICV administration of GnIH during photophase decreased female-mounting behavior and penile insertion, but it did not affect ambulatory behavior	Johnson et al. (2007)
		male and female sheep, mice, cynomolgus macaques	Chronic ICV administration of GnIH did not affect sexual behaviors measured in primates or ewes; Administration during scotophase in rats did not affect sexual behavior	Clarke et al. (2012)
		male Pekin ducks	ICV administration of GnIH depressed plasma LH and stimulated feeding. 48 h of fasting depressed LH, induced immidiate early gene expression in GnIH-ir cells	Fraley et al. (2013)
		female Syrian hamsters	Chronic ICV administration of GnIH reduced sexual motivation and vaginal scent marking but not lordosis behavior	Piekarski et al. (2013)
		male and female white crowned sparrows and male Japanese quail	RNAi of GnIH gene increased breeding and territorial-like vocalizations in sparrows. In quail, it induced sexual and aggressive behaviors, and GnIH administration suppressed these behaviors	Ubuka et al. (2013)
		male Japanese quail	GnIH inhibits sexual behavior by activating aromatase and increasing neuroestrogen synthesis	Ubuka et al. (2013)
	Response to environment	male Rufous-winged sparrow	During monsoons (a cue for breeding), sparrows had fewer, less densely-labeled GnIH-ir cells and fibers than birds caught before the monsoons	Small et al. (2008)
		male and female European starling	Both sexes differed similarly in GnIH-ir cell abundance depending on whether they obtained a nest box or not, and whether they were incubating or not	Calisi et al. (2011)
Phylogeny:		Review paper	Phylogenetic tree of Rfamides invoved in seasonal reproduction in vertebrates	Grieves et al. (2008)
		Review paper	Compilation of amino acid sequences of GnIH and its homologues and their hypophysiotropic actions	Tsutsui and Osugi (2009)
		Review paper	Compilation of amino acid sequences of GnIH and its homologues and their function in human, monkey, rat, hamster, bovine, quail, sparrow, amphibian, fish	Tsutsui (2010)

At time of hatching, androgens are high in circulation (Ottinger and Bakst, 1981) and plasma luteinizing hormone and follicle stimulating hormone are low (Tsutsui and Ishii, 1985). These events led Ubuka et al. (2003) to posit that GnIH may play an important role in gonadotropin regulation around hatching, serving as part of a negative feedback system in which gonadal steroid production activates GnIH, and subsequently GnIH inhibits gonadotropin release.

Expression of GnIH can be detected at all embryonic stages of the zebrafish as well as during early development and hatching (Zhanga et al., 2010), however, the expression patterns of GnIH receptors differ among the various stages, suggesting a changing role for GnIH and its receptors during the early development (Zhanga et al., 2010). Similarly in rats, GnIH expression varies in the hypothalamus of female rats during development (Maze et al., 2013). Between day 10 and day 60 post-partum (PND10-PND60), GnIH expression is most abundant on PND30. After PND30, expression declines during the transition into puberty. Maze et al. (2013) suggested that once the hypothalamus is fully developed, upregulation of GnIH may occur to inhibit reproductive function until puberty is reached.

GnIH can also be produced in the testes (McGuire and Bentley, 2010; Zhao et al., 2010). In contrast to the decrease in female rat hypothalamic expression observed by Maze et al. (2013) during early development, Anjum et al. (2012) reported a significant increase in GnIH expression in the Leydig cells of mouse testes from the prepubertal to the pubertal period. A decrease in GnRH receptor expression has also been reported during this same time period, coinciding with a decrease in testosterone synthesis. This down-regulation may be related to or be the result of a concomitant increase in GnIH expression (Anjum et al., 2012). Anjum et al. (2012) suggested that GnIH may play a role in spermatogenesis during puberty, whereas later in life, it may inhibit GnRH receptor expression.

To gain a better understanding of how the role of GnIH changes with ontogeny, more in-depth profiling and manipulation over the course of development and senescence is needed. How and why does GnIH change over development, and what are the consequences? How might these changes in GnIH be shaped by gene-environment interactions? Changes in not only GnIH gene expression but also in processes such as protein production, dendritic abundance and placement, receptor expression, and parental, epigenetic and environmental effects can give a more complete picture of how the GnIH system develops within an individual. Manipulating the GnIH system and assessing the resulting behavioral manifestations provides an even more powerful way to understand the role of GnIH during particular stages of ontogeny.

3. Ultimate views: adaptive and evolutionary perspectives

3.1. Function: how does GnIH enhance survival and reproduction?

Much of evolution is thought to proceed due to natural selection, which hones the function of traits that increase survival and reproduction in the current environment. By this process, adaptive traits are inherited by offspring, who will then have an increased propensity to survive and reproduce because of these traits. This powerful phenomenon not only acts on well-known morphological traits such as finch beaks, peppered moth coloration, and snake pattern mimicry but can also operate on physiological traits (McGlothlin et al., 2010; Dantzer et al., 2013).

Only relatively recently has it become apparent that GnIH affects behavior (Bentley et al., 2006a). Since this report, experimental and observational studies have examined the relationships of GnIH with stress, feeding, and reproductive behaviors of various vertebrates and how these relationships are affected by the environment. These studies are reviewed below.

3.1.1. Survival and behavior

3.1.1.1. Stress. Unpredictable and uncontrollable events – stressors – can disrupt physiological homeostasis in ways that can be detrimental to an organism's ability to survive and reproduce. Because of this potential problem, organisms have evolved mechanisms to cope with such disruptive, "stressful" events and to restore homeostasis. This process of achieving stability through physiological or behavioral change, termed allostasis (Sterling and Eyer, 1988), was later expanded upon to incorporate more ecologically relevant data in McEwen and Wingfield's Allostatic Model (2003b). Building on the strengths of this model, Romero et al. (2009) proposed the Reactive Scope Model to better formulate testable predictions for how physiological systems will respond to stressful events and their ultimate impact on the organism. These frameworks highlight the complexity of how an animal survives and reproduces in the wake of stressful events and emphasizes that multiple external (environmental) and internal (physiological) processes need to be examined.

Stress frequently has a negative impact on reproduction and reproductive behaviors. This inhibition can be adaptive by discouraging breeding in the short term, when attention would be better spent on evading a predator than on courting a mate. Inhibiting reproduction in the face of stress may also prove beneficial in the long term when raising offspring in a hostile environment might induce greater costs than benefits for the parent.

As reviewed above, GnIH, like stress, can inhibit reproduction by decreasing reproductive behaviors and reproductive hormones circulating in the blood. This similarity in function inspired the hypothesis that perceived stress inhibits reproduction via GnIH (Calisi et al., 2008). Stress in male and female house sparrows (*Passer domesticus*), as induced by confinement for 1 h in a bag, can increase the number of hypothalamic cells immunoreactive for the GnIH protein (Calisi et al., 2008). House sparrows are a seasonally breeding species, and this relationship was found only during their reproductive period. In contrast, no relationship was found between this type of confinement stress and the number of GnIH-positive cells during the non-breeding period, suggesting this system may only be functional during a time important for specific reproductive behaviors (Calisi et al., 2008). The generality of this relationship was confirmed in male Sprague–Dawley rats; acute and chronic immobilization stress led to an increase in hypothalamic GnIH-positive cells (Kirby et al., 2009). Additionally, Kirby et al. (2009) demonstrated that the removal of glucocorticoids – hormones that increase in circulation to aid in the stress response – via adrenalectomy blocked this response. Both Kirby et al. (2009) and Calisi et al. (2010) reported that GnIH and receptors for glucocorticoids were co-expressed in these cells, providing one type of infrastructure by which the stress response could negatively impact reproduction and associated behaviors via effects on GnIH.

How GnIH responds to stressors in a natural setting is currently unknown. Climate change, urbanization, pollution and other stressors are associated with negative effects on reproduction in various species. Is GnIH involved in the communication of and response to such events? If so, could manipulation of the GnIH system negate the inhibitory effects of stress on reproduction? As reviewed in the previous section, GnIH can change over the course of development. How do different types of stress experienced at different stages of development affect GnIH, and how might this interaction affect the reproductive system? This intricate knowledge of how the brain responds to such stressors will increase our understanding of how these stimuli affect organism survival and reproduction and potentially how we can abrogate their detrimental effects.

3.1.1.2. Food. While some people claim they "live to eat," most organisms must eat to live, and GnIH has been hypothesized to play a role in balancing aspects of feeding with reproduction (Tachibana et al., 2005; Johnson et al., 2007; Klingerman et al., 2011; Clarke et al., 2012). Regulation of food and water intake is essential for survival and reproduction. For example, food restriction can decrease the release of LH and sex steroids (Richard-Yris et al., 1987; Liang and Zhang, 2006; Lynn et al., 2010) and decrease the survival rate of offspring (Liang and Zhang, 2006). Administration of sex steroids can in turn reduce food intake (Jaccoby et al., 1995; Snapir et al., 1983). This phenomenon and the fact that the PVN, home to GnIH cells in birds, is also involved in avian feeding regulation (Denbow and Sheppard, 1993) inspired Tachibana et al., (2005) to test the hypothesis that the inhibition of sex steroid release by GnIH induces feeding in birds.

Tachibana et al., (2005) administered an intracerebroventricular (ICV) injection of GnIH in red jungle fowl chicks (*Gallus gallus*). Compared with controls, GnIH significantly stimulated food intake. ICV administration of GnIH also increased food uptake in male Sprague–Dawley rats (Johnson et al., 2007) and Pekin drakes (*Anas platyrhynchos domestica*) (Fraley et al., 2013). ICV administration of LPLRFamide, which exhibits a degraded C-terminus of GnIH, did not stimulate feeding behavior, leading the authors to posit the importance of the N-terminus of GnIH in feeding behavior (Tachibana et al., 2005). GnIH projects to what are considered major appetite-regulating regions (Qi et al., 2009; Ubuka et al., 2009a; Fraley et al., 2013) and Tachibana et al. (2008) reported that GnIH-induced feeding may be mediated by the opioid mu-receptor.

Klingerman et al. (2011) explored the connection between GnIH, reproduction, and feeding behaviors in female Syrian hamsters (*M. auratus*). In nature, there are times in which organisms may be faced with the choice of either foraging for food or engaging in courtship. As GnIH has been reported to inhibit courtship behaviors and stimulate feeding behaviors, could GnIH serve as a dynamic switching mechanism between feeding and reproduction? Klingerman et al. (2011) reported that food restriction activated GnIH, with activation measured via the quantification of double-labeled cells for the immediate early gene (IEG) FOS and the GnIH peptide. This activation was associated with increased food hording and decreased reproductive behavior. However, although the number of double-labeled cells and percentage of cells co-labeling for GnIH and FOS increased, the actual number of cells immunoreactive for GnIH (GnIH-ir) decreased with food restriction. Interestingly, and unlike the results of Tachibana et al. (2005) and Johnson et al. (2007), hamsters did not ingest more food, but the appetitive behavior of food hording increased in concert with the increased activation of GnIH cells. Vaginal scent marking, a type of proceptive female behavior, decreased with food restriction and was negatively correlated with GnIH cell activation. However, as the authors note, these data do not prove that GnIH *causes* these behavior changes.

In 2012, Clarke et al. manipulated GnIH in multiple species to better understand the connection between GnIH and sexual and feeding behaviors. Consistent with the results reported by Tachibana et al. (2005) and Johnson et al. (2007), ICV administration of GnIH blocked the estrogen-induced LH surge in female sheep and increased food intake in male and female sheep, mice, and cynomolgus macaque monkeys. Administration of GnIH, combined with its associated increased food intake, did not appear to affect energy expenditure or sexual behavior in sheep or rats. Interestingly, while Bentley et al. (2006a) and Johnson

et al. (2007) reported *rapid* negative effects of GnIH on sexual behavior, Clarke et al. (2012) found no pronounced *long term* effects of GnIH administration. The negative relationship between feeding and a GnIH-induced LH surge necessary for reproduction led Clarke et al. (2012) to propose GnIH as a molecular switch mediating a preference for food over reproduction and vice versa. However, many questions remain as to if and how GnIH directly regulates the balance between feeding and reproductive behaviors and how this operates in various temporal dimensions.

3.1.2. Reproductive behavior

Many studies have explored the interactions between GnIH and the reproductive axis (see previous list of reviews; Fig. 1). However, much less is known regarding how changes in GnIH manifest in reproductive behaviors and how, in turn, the environment affects GnIH. In 2006(a), Bentley et al. measured the effects of GnIH on the sexual behavior of adult female white crowned sparrows (*Z. leucophrys gambelii*). Female sexual receptivity in many species of passerine birds is indicated by a copulation solicitation display in response to the male song. This display is characterized by the raising of the tail and head, fluttering of the wings, and a copulation solicitation-associated vocalization. When the authors administered what was determined to be a physiologically relevant dose of GnIH (200 ng in 4 μL saline) ICV, solicitation displays significantly decreased within the first 30 min compared with saline controls. No differences were observed in locomotor activity as measured by the number of perch hops between the experimental and control groups, suggesting the negative effects of GnIH on sexual behavior were specific, not an effect of debilitation or broadly decreased motivation.

The following year, Johnson et al. (2007) reported that ICV administration of RFRP-3 suppressed sexual behavior but not ambulatory behavior in male Sprague–Dawley rats. In this study, the rats were injected with either a 100 ng/3 μL or 500 ng/3 μL dose of RFRP-3. Like Bentley et al. (2006a), sexual behaviors were measured within the first 30 min. The higher dose of RFRP-3 yielded significant decreases in female-mounting behavior and penile insertion during the photophase (light part of light:dark cycle) but not the scotophase (dark part of light:dark cycle). Affinity-purified anti-GnIH, intended to suppress the effects of RFRP-3, significantly increased sexual behaviors compared with that observed in vehicle controls.

Similarly, Ubuka et al. (2013) injected GnIH via ICV administration in photostimulated (reproductively active) male and female white crowned sparrows and male Japanese quail (*C. Japonica*). They also used RNA interference (RNAi) of the GnIH gene to suppress GnIH mRNA translation. Upon examination of behaviors 2 days later, they found that the use of GnIH RNAi increased breeding- and territorial-like vocalizations in the sparrows. In quail, GnIH RNAi induced sexual and aggressive behaviors when measured 1 day later, whereas GnIH administration suppressed these same behaviors at the same time point. Recently, Ubuka et al. (2014) reported that GnIH can inhibit male sexual behaviors in quail by directly activating aromatase, increasing neuroestrogen synthesis beyond its optimum concentration for the expression of sexual behaviors.

As mentioned previously, Clarke et al. (2012) did not find significant effects of GnIH on sexual behavior in non-human primates (number of mounts), ewes (time spent near males and receptivity), or rats (number of mounts, intromissions, ejaculation and latencies for these events to occur). Unlike the single injection(s) of GnIH administered centrally by Bentley et al. (2006a) and Johnson et al. (2007), in the study by Clarke et al. (2012), primates received a continuous infusion of GnIH over 9 days, and ewes received a bolus followed by a continuous infusion of GnIH for 20 h. In rats, sexual behavior was measured during scotophase, a time during which Johnson et al. (2007) found sexual behaviors to be unaffected by GnIH administration. In contrast, Piekarski et al. (2013) found that a chronic central infusion of GnIH over several days reduced sexual motivation and vaginal scent marking in female Syrian hamsters but not lordosis behavior. The authors point out that in this case, proceptive behaviors ('appetitive behaviors') were affected without affecting the 'consumatory components' of sexual behavior (lordosis). Taken together, the results from Bentley et al. (2006a), Johnson et al. (2007), Clarke et al. (2012) and Piekarski et al. (2013) highlight the importance of GnIH dosage, timing of administration, and classification of different types of sexual behaviors when measuring and reporting the effect of GnIH on sexual behavior. What is emerging is a picture of acute inhibition but possible long-term effects of GnIH on reproductive behavior, though further characterization and manipulation of this system is needed to provide a greater understanding.

3.1.3. Response to environment

Organisms have evolved to survive and reproduce in their natural environment. Understanding how changes in the environment

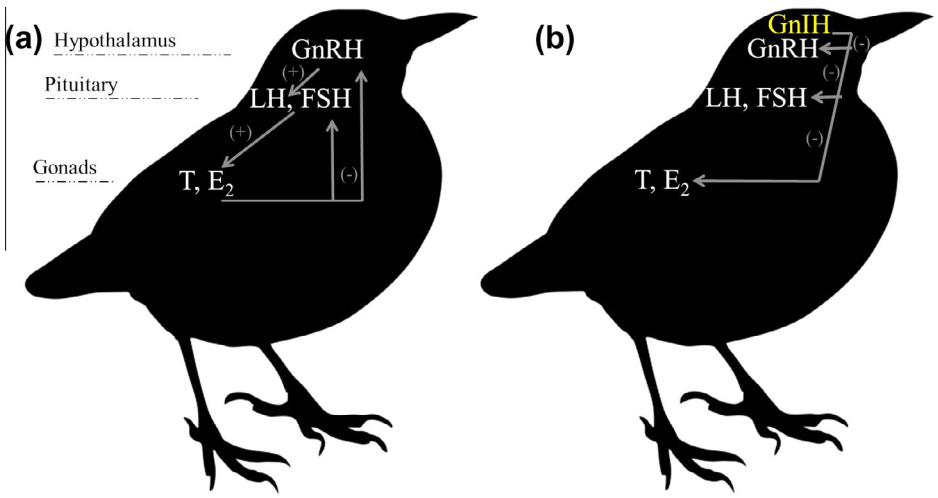

Fig. 1. A classic, simplified schematic of the hypothalamic–pituitary–gonadal (HPG), or Reproductive Axis. (a) Activation of the HPG axis by GnRH. (b) Inhibition of the HPG axis by GnIH.

(for example, seasonal, social and ecological changes) can affect characteristics such as GnIH function can aid in elucidating how the brain integrates and processes information and how this information influences behavior. Very few studies have examined the effects of the environment on GnIH. Seasonal comparisons of GnIH/RFRP in seasonally-breeding passerine birds and mammals have revealed that GnIH appears to be up-regulated in the brain during the breeding season as compared with the non-breeding season (Bentley et al., 2009a,b; Calisi et al., 2010; Mason et al., 2010; Tsutsui et al., 2010a). A reproductive inhibitory hormone that increases during a time of reproduction may seem counter-intuitive, but it is thought that GnIH may modulate or 'pause' the HPG axis in response to environmental perturbations during this time. Once birds are no longer breeding, there may not be a need for this modulation, and thus GnIH becomes down-regulated in the brain. However, the seasonally breeding marsupial, the brush-tail possum (*Trichosurus vulpecula*), exhibits a 2-fold increase in the RFRP cell body number during the nonbreeding season (summer) compared to the breeding season (winter) (Harbid et al., 2013). Sheep also seem to downregulate RFRP during the breeding period (Smith et al. 2008).

Small et al. (2008) examined the effects of monsoons on GnIH expression in male Rufous-winged sparrows (*Aimophila carpalis*). These sparrows live in the Sonoran desert and breed after summer rains. It was previously reported that rainfall regulates LH in this species (Deviche et al., 2006; Small et al., 2007), leading researchers to explore how this may relate to GnIH expression. During the monsoons, a period of high LH levels, the sparrows had fewer, less densely-labeled GnIH-ir cells and fewer GnIH cell fibers than birds caught before the monsoons (a period of low LH levels). No GnIH fibers were found to exist in the median eminence, suggesting that in this case, GnIH may not directly inhibit LH secretion from the pituitary. However, Small et al. (2008) posited that GnIH may inhibit GnRH activity, reducing LH production. GnIH-ir fibers decreased in the POA (where GnRH is found) during the monsoon, lending support to this notion (Small et al., 2008).

In 2011, Calisi et al. reported that the number of GnIH-ir cells in male and female European starlings (*Sturnus vulgaris*) was associated with social rank and the ability to procure a nest box during the breeding season (Calisi et al., 2011). European starlings are a socially monogamous, obligate cavity-dwelling species, meaning that in order to breed, starlings require a cavity in which to build their nests, lay and incubate their eggs, and care for their chicks. Because nesting cavities can be a limited resource in the wild, Calisi et al. (2011) limited the number of potential cavities by using nest boxes within large, naturalistic outdoor aviaries. Limiting this resource provided social conflict in which starlings would aggressively interact, the victors gaining use of nest boxes and the resulting reproductive opportunity. Although starlings sometimes partake in extra-pair copulations in the wild, paternity analyses within this population confirmed social parents of chicks to be the biological parents. Calisi et al. (2011) discovered that there were fewer GnIH-ir cells in the brains of males and females that outcompeted others for nest boxes as compared to their out-competed counterparts. Whether this difference in cell number was a response to obtaining a nest box or existed prior to obtainment – possibly influencing competitive behaviors – is unknown. However, less inhibition to the reproductive axis at this time could be adaptive in either situation: acquiring a mate and nest box and/or commencing breeding as soon as a mate and nest box were obtained.

Later in the breeding season, when the birds with nest boxes had begun egg incubation, GnIH-ir cells in males and females significantly increased in number compared with earlier in the season when the birds had first acquired boxes. GnIH-ir cells were twofold more abundant in incubating birds compared with birds without boxes at this time (Calisi et al., 2011). No difference in the GnIH-ir cell number was detected between these sampling time points in birds without a nest box. Calisi et al. (2011) hypothesized that this increase in the number of GnIH-ir cells might be related to a decrease in testosterone and sexual behaviors on which parental care is dependent. Because the social parents and owners of the nest boxes were confirmed to be the genetic parents of the offspring, this inspires thought as to the adaptive value of GnIH function.

The results of Calisi et al. (2011) demonstrated potential ecological (having a nest box versus not having a nest box) and social (competition for nest boxes and parental care) effects of GnIH-peptide expression. Authors are currently exploring the relationship between GnIH and parental care in birds and mammals in greater depth using various types of experimental manipulations (Calisi et al., 2013). One such manipulation involved using the RFRP-receptor antagonist RF9 (Pineda et al. 2010) to block the effects of GnIH in birds *in vivo*. However, RF9 administered intracerebroventricularly or sub-cutaneously via an osmotic mini-pump in multiple avian species proved an unreliable, problematic tool to manipulate GnIH function (Calisi et al., 2013; Jesse Krause and John Wingfield, *personal communication*). Other methods of GnIH manipulation *in vivo* are currently being explored. It is essential to be able to manipulate the system to measure the effects of GnIH on natural behaviors. Better tools must be developed and utilized to permit the manipulation of GnIH in an animal's natural environment to discover how environment can affect GnIH and its function.

3.2. Phylogeny: how did GnIH evolve?

Comparing the form and function of a neurohormone such as GnIH across species in a phylogenetic framework can help us to understand the evolution of GnIH. While Tinbergen's question of function captures how natural selection may have acted upon the GnIH system, his question of phylogeny considers evolutionary history. For example, when in evolutionary history did GnIH first emerge? When might a mutation have occurred in the GnIH sequence, creating variants between species? What constraints exist on the system due to the early evolutionary development of GnIH? Understanding phylogenetic aspects of a neurohormone yields a greater understanding of the constraints and momentum shaping its role in present systems.

Greives et al. (2008), Tsutsui and Osugi (2009) and Tsutsui (2010) fueled the conversation regarding the phylogenetic origins of GnIH by comparing and contrasting aspects of GnIH and its homologs among vertebrates. Greives et al. (2008) produced a phylogenetic tree of the RFamide peptide family, which included the GnIH group, and explored the role of such RFamides in seasonal reproduction. Tsutsui and Osugi (2009) and Tsutsui (2010) compiled the amino acid sequences of GnIH and its homologs (LPXRFamide peptides, with X = L or Q) and their known functions in humans (Ubuka et al., 2009a), monkeys (Ubuka et al., 2009b), rats (Hinuma et al., 2000; Ukena et al., 2002), hamsters (Kriegsfeld et al., 2006), bovines (Fukusumi et al., 2001; Yoshida et al., 2003), quail (Tsutsui et al., 2000; Satake et al., 2001), sparrows (Osugi et al., 2004), bullfrogs (Koda et al., 2002; Ukena et al., 2003a), European green frogs (Chartrel et al., 2002), and goldfish (Sawada et al., 2002). What is brought to light is that GnIH and its homologs appear to be conserved for the most part in form and function as regulators of pituitary hormone release (Ukena and Tsutsui, 2005; Tsutsui and Ukena, 2006; Tustsui, 2009), extending beyond control of gonadotropins. GnIH homologs in frogs, fGRP and fGRP-RP-2, can stimulate the release of growth hormone and prolactin (Ubuka et al., 2003; Koda et al., 2002), and fish

homologs gfLPXRFa-1, -2, and -3, can stimulate the release of growth hormone and gonadotropins but not prolactin (Amano et al., 2006). When the biologically active GnIH homolog RFRP-1 (found in some mammals) was administered via an ICV injection into rats, it increased prolactin release (Hinuma et al., 2000). When RFRP-3, another GnIH homolog found in mammals, was administered in the same fashion to rats, it decreased circulating LH (Johnson et al., 2007; Murakami et al., 2008), as did an ICV injection of GnIH in hamster (Kriegsfeld et al., 2006). GnIH also reduces circulation of LH in birds (Tsutsui et al., 2000; Bentley et al., 2009a,b). These data and more (Johnson et al., 2007; Clarke et al., 2008; Murakami et al., 2008; Tachibana et al., 2005) yield evidence that GnIH and RFRP-3 are functional homologs, presenting possible instances of evolutionary divergence of function (Tsutsui, 2010) and fueling future investigations into RFRP subtypes.

4. Concluding thoughts

Over the 14 years since the discovery of GnIH, we have acquired a great deal of knowledge concerning its mechanistic properties relative to our understanding of its behavioral manifestations (Table 1). There is evidence that GnIH expression can change over the course of development (Zhanga et al., 2010; Anjum et al., 2012; Maze et al., 2013), but at this point, we can only speculate as to why it does this. GnIH is associated with behaviors important for survival and reproduction, such as the stress response (Calisi et al., 2008; Kirby et al., 2009), feeding behaviors (Tachibana et al., 2005; Johnson et al., 2007; Klingerman et al., 2011; Clarke et al., 2012), and social, environmental, and reproductive behaviors (Bentley et al., 2006a; Small et al., 2008; Calisi et al., 2011; Ubuka et al., 2013; Piekarski et al., 2013; Ubuka et al., 2014). We also have information regarding changes in the structure and function of this neuropeptide in various taxa (Greives et al., 2008; Tsutsui and Osugi, 2009; Tsutsui et al., 2010c). The use of an integrative framework like Tinbergen's can reveal patterns not evident from studies that focus on just one question (Fig 2). For all questions of mechanism, ontogeny, function and phylogeny, there exists both similarities and differences in GnIH form or function within and among species. An integrative, comparative approach to studying this hormone may reveal reasons for this. For example, looking at the adaptive function and evolutionary (phylogenetic) patterns of GnIH variation may identify examples of convergence and divergence in function that are not otherwise visible.

We have just begun to understand the role of GnIH in vertebrate behavior. As we forge ahead, some pressing questions and lines of investigation to consider include: is the action of GnIH organized at birth and/or activated by the environment? How does organization and activation affect the role of GnIH throughout the life of an organism? How does GnIH respond to changes in the natural environment in which an organism evolved (e.g., stressful events, social and ecological changes) or in response to human encroachment (e.g., urbanization, pollution, climate change) during development, adulthood, and senescence? Does GnIH directly regulate the balance between feeding and reproductive behaviors? If so, how is this regulation affected over a lifespan and in response to environmental perturbations? What are the immediate versus long-term effects of GnIH activation? Does GnIH, in regards to all previous questions, activate and function similarly in all vertebrates? If not, what was the cause of the divergence in function, and what can this reveal about the role of GnIH in behavior?

Ideally, future observations and manipulations should be conducted in the organism's natural environment – the environment in which they (and GnIH) have evolved to survive and reproduce. By removing an organism from such an environment, we may unknowingly alter the development and function of such neuroendocrine factors, and thus our search for the true function of these factors may be ineffective (Calisi and Bentley, 2009). However, studies in the natural environment are not always possible, and model organisms in controlled environments can be powerful

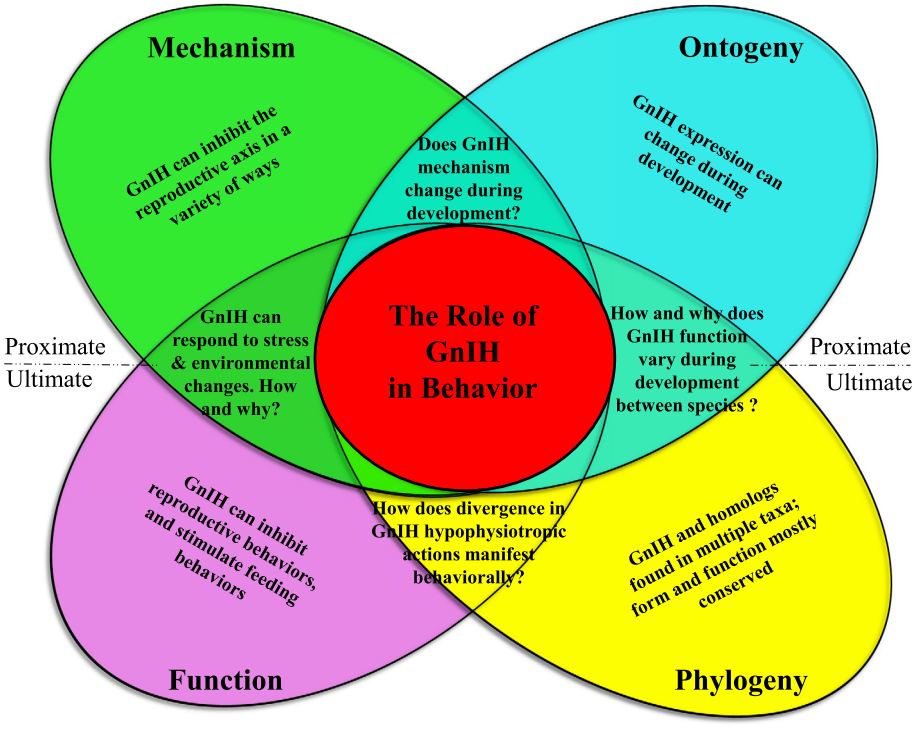

Fig. 2. A Venn diagram illustrating the intersection of inquiry projected by Tinbergen's framework. Consideration of both proximate and ultimate levels of analyses can aid in directing research and interpretation of resulting data to elucidate any hormonal phenotype, such as that of GnIH, and its role in behavior.

investigative tools. Indeed, controlling for as many environmental variables as possible can facilitate an accurate interpretation of the resulting data (Calisi and Bentley, 2009). By synthesizing our knowledge of GnIH gathered from both lab and field environments over the course of an organism's development, we will deepen our understanding of its purpose and function.

Due to the integrative nature of investigations needed to answer these questions, we should be cognizant of Tinbergen's framework to help better direct future research of GnIH. It is important to understand not only how a neurohormone works instantaneously, but also how it works over the course of an individual's life, how environmental perturbations during the individual's life (and even prior to it) affect such functioning, how selection has acted upon the neuroendocrine system in question, and the evolutionary history of that system. More in-depth profiling and manipulations of GnIH over the course of these stages as well as additional comparative investigations of structure and function in a variety of taxa will bring us closer to gaining a full understanding of the role of GnIH.

Increasingly, scientists exhibit a great deal of specialization in their respective fields. This focus has undoubtedly yielded profound advancements. However, in his 1963 work entitled "On aims and methods in Ethology," Nikolaas Tinbergen stated that "It just is a fact that we are still very far from being a unified science, from having a clear conception of the aims of the study, of the methods employed and of the relevance of the methods to the aims." Arguably, today, we still lack efficient integration of knowledge generated by different behaviorally-relevant fields of study. Hard at work are molecular biologists, developmental biologists, animal behaviorists, and evolutionary biologists, but the secrets to fully comprehending any particular phenotype – be it behavioral, hormonal, or anything else – lie in connecting these sub-fields. As we push forward, we should engage, discuss and collaborate beyond our subfields to promote a more well-rounded, integrative study of physiology and behavior.

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