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Wesley C. Hymer
The Pennsylvania State University

Mary J. Kennett
The Pennsylvania State University

Samir K. Maji
IIT Bombay

Kristin L. Gosselink
Burrell College of Osteopathic Medicine

Gary E. McCall
University of Puget Sound

See next page for additional authors

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Review Article

Bioactive growth hormone in humans: Controversies, complexities and concepts

Wesley C. Hymer[a], Mary J. Kennett[b], Samir K. Maji[c], Kristin L. Gosselink[d], Gary E. McCall[e], Richard E. Grindeland[f], Emily M. Post[g], William J. Kraemer[g,*]

[a] Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA 16802, United States of America
[b] Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, University Park, PA 16802, United States of America
[c] Department of Biosciences and Bioengineering, IIT Bombay, Powai, Mumbai 4000076, India
[d] Department of Physiology and Pathology, Burrell College of Osteopathic Medicine, Las Cruces, NM 88001, United States of America
[e] Department of Exercise Science Exercise and Neuroscience Program, University of Puget Sound, Tacoma, WA 98416, United States of America
[f] Life Science Division, NASA-Ames Research Center, Moffett Field, CA 94035, United States of America
[g] Department of Human Sciences, The Ohio State University, Columbus, OH, 43210, United States of America

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ABSTRACT

Objective: To revisit a finding, first described in 1978, which documented existence of a pituitary growth factor that escaped detection by immunoassay, but which was active in the established rat tibia GH bioassay.
Methods: We present a narrative review of the evolution of growth hormone complexity, and its bio-detectability, from a historical perspective.
Results: In humans under the age of 60, physical training (i.e. aerobic endurance and resistance training) are stressors which preferentially stimulate release of bioactive GH (bGH) into the blood. Neuroanatomical studies indicate a) that nerve fibers directly innervate the human anterior pituitary and b) that hind limb muscle afferents, in both humans and rats, also modulate plasma bGH. In the pituitary gland itself, molecular variants of GH, somatotroph heterogeneity and cell plasticity all appear to play a role in regulation of this growth factor.
Conclusion: This review considers more recent findings on this often forgotten/neglected subject. Comparison testing of a) human plasma samples, b) sub-populations of separated rat pituitary somatotrophs or c) purified human pituitary peptides by GH bioassay vs immunoassay consistently yield conflicting results.

1. Introduction

The very long history of growth hormone (GH) research, encompassing approximately 80 years, has often produced controversial results. A pituitary growth factor that escaped detection by immunoassay, but had GH activity in the established rat tibia bioassay, was first described in 1978 [1]. We utilized common search engines of PubMed, Web of Science, and Google Scholar along with insights by the different investigators who all have a history with research on GH. The purpose of this review is to revisit this puzzling and controversial result.

Our review is driven by three concepts:

Concept #1. A subpopulation of somatotrophs respond to signals from hypothalamic nuclei and secrete bioactive GH-like (bGH-L) form (s) that are poorly recognized by GH antisera directed against the native (22 kDa) form.

Concept #2. Peripheral and/or proprioceptive signals (neural afferents) produced from hind-limb muscles regulate pituitary bGH-L content and release.

Concept #3. Human pituitary glands contain a peptide, not structurally related to hGH, which is active in the tibia bioassay.

2. Defining bioactive growth hormone

How do we define bGH-L? We restrict our definition to in vivo growth responses of the test organism upon injection. These include either a) widening of tibial epiphyseal growth plates in the hypophysectomized rat (tibial line assay) or b) anabolic responses in hypophysectomized rats (muscle and body weight change). The topic of GH control of lipid/glucose metabolism, although strictly defined as a bGH-L endpoint, is considered to be outside the scope of this review. Our

* Corresponding author at: Department of Human Sciences, The Ohio State University, A054 PAES Building, 305 Annie & John Glenn Avenue, Columbus, OH 43210, United States of America.
E-mail address: kraemer.44@osu.edu (W.J. Kraemer).

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strict definition therefore excludes consideration of a large body of information generated from experiments using in vitro cell based assays even though it can be easily argued that such assays do indeed measure a biological response.

2.1. Early history

The early history (1940's) of GH isolation from pituitary tissues of many mammalian species was described in a review by Papkoff and Li in 1962 [2]. In 1956 C.H. Li proposed that a better name for the hormone would be “metabolic hormone” [3]. Even today, there is no direct, simple answer to the question of how one should measure the hormone's biological activity. Clearly, this issue is not new.

In 1978, pioneering studies by Ellis et al. [1] showed that treatment of rat pituitary extracts with GH antisera failed to neutralize this biologically active factor. Further, their experiments showed that results from biological and immunological assays did not correlate. The concept of a dichotomy between bioactive (bGH-L) and immuno-reactive (iGH) hormone evolved from these early experiments; a dichotomy which was confirmed by others shortly thereafter. Although the underlying reason(s) remained unknown, structural modification(s) of the native, 191 amino acid, 22 kDa form of the hormone during its' release from the pituitary was proposed as a mechanism to explain the dichotomy.

During this early period, the three most often used growth bioassays were a) the weight gain assay in the plateaued female rat; b) the weight gain assay in the immature hypophysectomized rat and c) the tibia test. To this day, practical considerations such as animal cost and assay sensitivity (and therefore total amount of hormone required) seriously impact bioassay choice. The tibial assay is especially useful for measuring bGH-L concentrations in human plasma. Assay sensitivity is relatively high (~3–5 μg), small sample volumes can be tested, and the assay can be done in a relatively short (4 day) period. While it is often criticized as being labor intensive, costly and lengthy, the authors of this review argue that the assay yields information that is simply not obtainable any other way.

Examples of growth responses obtained using the bGH-L assays described in this review are related to a) the tibial assay (Fig. 1) and weight/bone growth responses after implantation of dispersed pituitary cells into the brain ventricles of immature hypophysectomized rats [4,5].

Today's biologist may be unaware of bGH-L and its' implications; if an awareness does exist, it is likely that complications associated with in vivo bioassays (cost, assay sensitivities, sample size requirements etc.) discourage many from using them. The ready availability of recombinant GH, coupled with the ease (and specificity) of immunoassays, has contributed to the current situation; i.e. one in which a majority of GH investigators fail to see the need for use of in vivo bioassays in their research. As a result, considerations underlying the physiological and biochemical heterogeneity of GH molecule(s) in a plasma sample become secondary to the usual goal of generating an iGH signal.

3. Why is the dichotomy problem so complex?

In his 2009 review, Baumann [6] clearly identifies the answer: heterogeneity arising at the levels of the GH gene, mRNA splicing, post-translational processing and GH metabolism, all severely challenge our full understanding of what biologically active GH is and how one should measure it (Fig. 2). It should be noted that the Bauman review relates to peptidic GH; it is possible other peptides, unrelated to GH, may account for some of the activities depicted in Fig. 2. He defines additional confounding factors, viz. a) variants of hGH are not available in pure form; b) the possibility that biological activities of the variants may vary between species (e.g. rat and human) and c) the involvement and importance of GH binding proteins in GH metabolism- each of which directly contribute to the complexity problem. Baumann concludes “the heterogeneity of GH is one important reason for the notorious disparity among assay results…*and the biological significance of this heterogeneity remains largely unknown*” (italics added for emphasis).

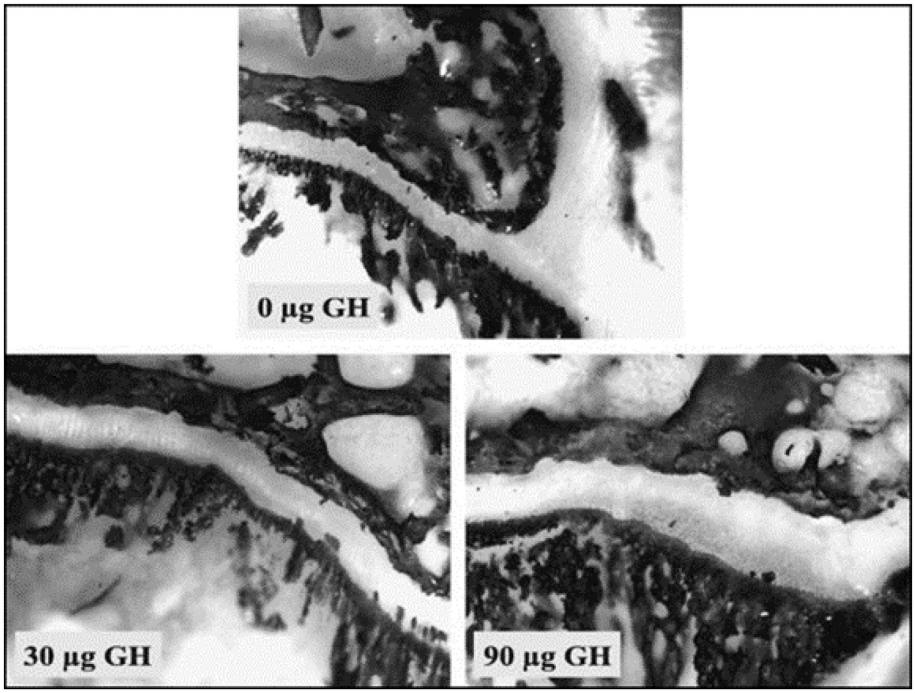


Fig. 1. The tibial assay is a fundamental bioassay to assess bGH-L activity in our studies reviewed in this paper. Widening of the tibial epiphyseal cartilage plate of the hypophysectomized rat after injection of a bovine GH standard in the 4 day assay.

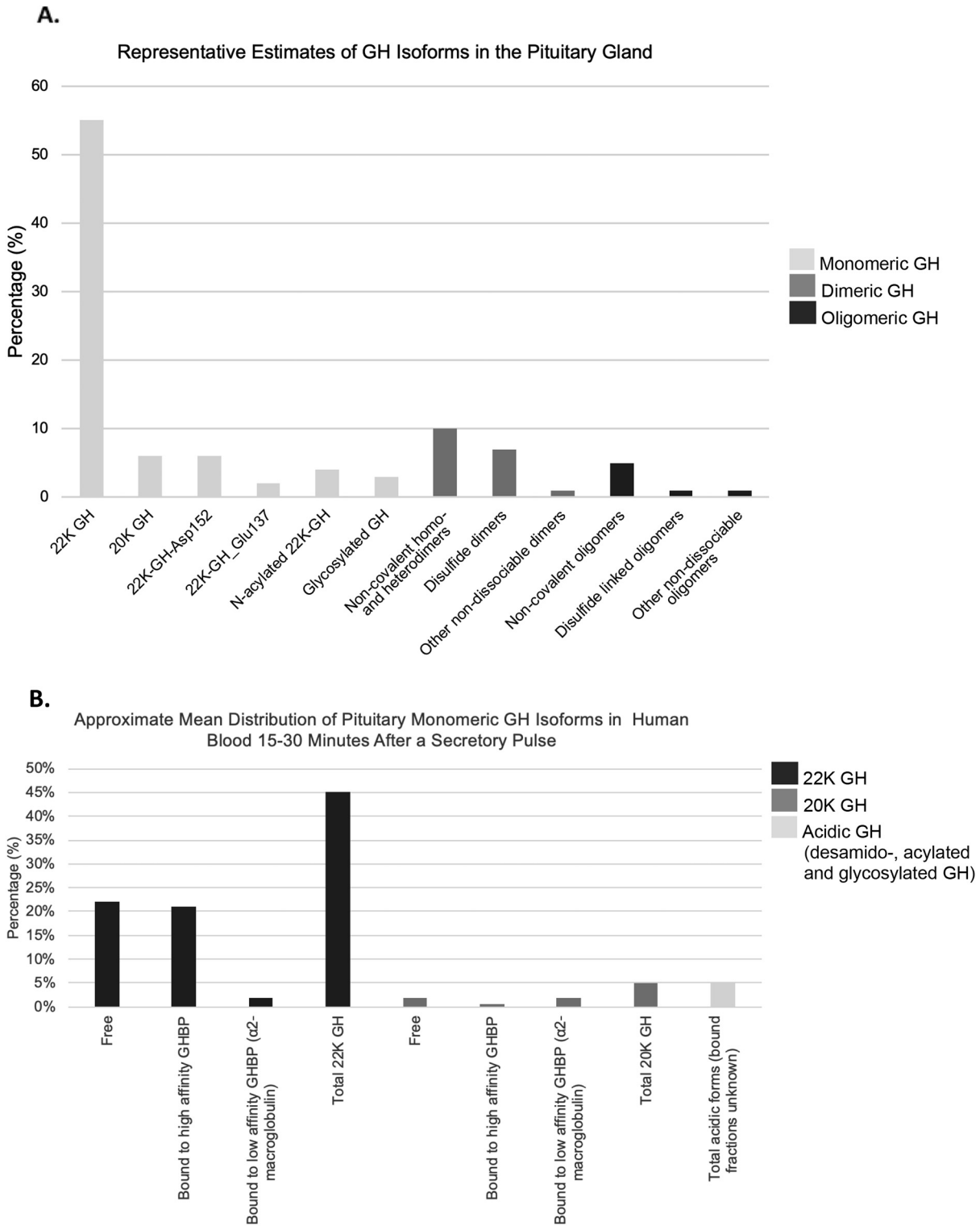
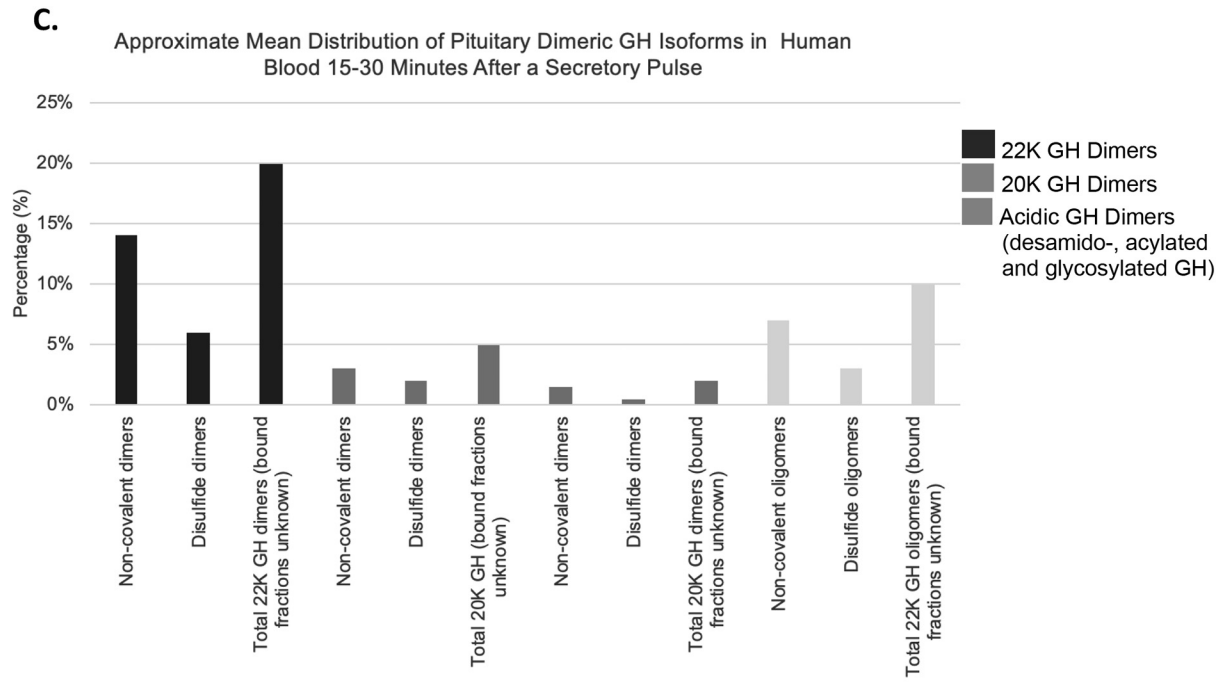


Fig. 2. An adapted presentation of data from Baumann (6) showing representative distributions of isoforms found in the A. pituitary gland and in the blood B. monomeric isoforms, C. dimeric isoforms and D. oligomer isoforms.

3.1. bGH-L AND iGH concentrations in rat and human anterior pituitary tissue

#1. Concentrations of bGH-L in human pituitary glands obtained at autopsy were reported by Parlow in 1974 [7]. Data, collected from 542 individuals, expressed in terms of a bovine GH standard, clearly reflect increased bGH-L concentrations in human pituitaries with advancing age. The hypophysectomized female rat body weight gain bioassay was used to generate these data.

#2. Results from Dr. Grindeland's laboratory indicate the following concentrations of pituitary bGH-L (tibia assay): Rat: 200–300 μg (iGH); 24–36 μg (bGH-L). Human: 4.4 mg (iGH); 0.53 mg (bGH-L). Medical texts suggest a range of 4–8 mg iGH/gland in the post mortem pituitary. They sometimes point out that GH containing secretory granules resist



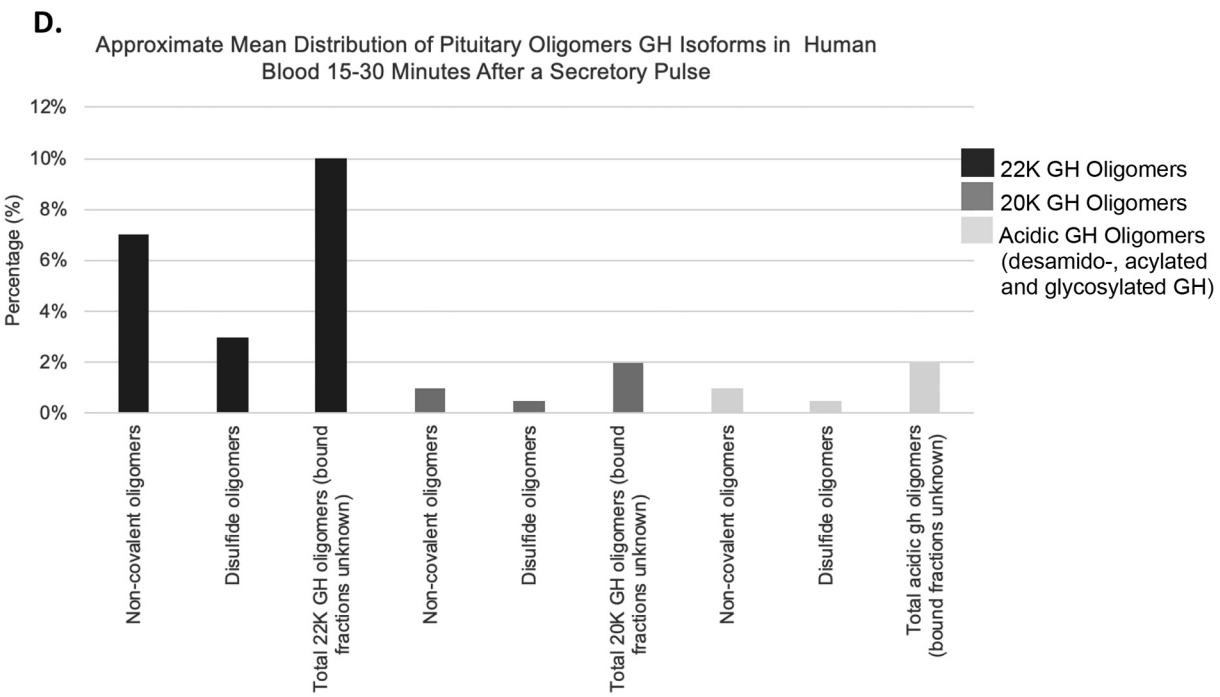


Fig. 2. (*continued*)

autolysis after death [8].

#3. Nearly 10% of the dry weight of the pituitary gland is thought to be GH. This concentration is ~800× more than any of the other pituitary hormones [9].

3.2. Section I. bGH-L in human plasma: recent studies

This topic was initially reviewed by McCall et al. in 2001 [10]. Results from 10 recent studies compare concentrations of bGH-L vs iGH in human plasma after exercise, fitness, age and voluntary muscle contraction. They reveal that bGH-L activity measures are consistently in the range of several thousand ug/L, while iGH measures in these same samples are only a few ug/L. This disparity is a function of the assay endpoint. In this context it is important to realize that preparations of GH, purified from pituitary glands of different species (e.g. human, bovine and murine), yield parallel dose response curves in the tibial assay. Similar tibial growth curve responses elicited by these standard preparations enable one to express biological potency in terms of a standard 22 kDa hormone. Results from the bGH-L data set below are expressed relative to a bovine GH standard (1.5 I.U.).

Of the 292 individuals comprising this data set, 190 were female. A majority (n = 226) were young (age range 19–27). Concentrations of bGH-L in the 938 plasma samples were several thousand ug/L of plasma (~2000–4000); but this range was extremely variable. 917 of the 938 plasma samples contained detectable amounts of bGH-L by tibial assay; however, 21 had concentrations that were undetectable (i.e. were at the

limit of assay sensitivity). These 21 samples were obtained from donors that averaged 81 yrs. old. Curiously, plasma samples from other 81 yr olds in this same cohort contained bGH-L concentrations in the range of 7000–9000 $\mu g \cdot L^{-1}$.

The primary test variable in 8 of the 10 studies was exercise of different types; i.e. large muscle group resistance exercise, small muscle group resistance exercise for ankle plantarflexion or aerobic exercise. In these 8 cases, the primary study objectives were to a) find out if bGH-L plasma concentrations changed after an acute exercise bout and b) how they related to iGH measures. In 4 of these 10 studies the acute exercise bout increased concentrations of plasma bGH-L, responses apparently related to previous exercise training; in 2 of them it decreased plasma bGH-L while in the remaining 4 trials, it had no effect on bGH-L relative to appropriate controls. Collectively, these results set up the hypothesis that exercise training primes the pituitary for increased bGH-L release in response to further exercise stressors. There is an important caveat in reaching conclusion(s) using this experimental approach; viz. the very presence of rat tibial widening after injection of a human plasma sample of course does not automatically imply that the sample is active on the bioassay target (bone) of the donor or other tissues.

- Hymer et al. [11]. A single bout of acute heavy resistance exercise in 24 yr old women significantly increased (2-4 ×) plasma concentrations of iGH, but not bGH-L. Fractionation of plasma by size exclusion chromatography showed that the increases were confined to classes of iGH in both dimeric (44 kDa) and monomeric (22 kDa)size range
- Kraemer et al. [12]. Chronic (6 months) bouts of heavy resistance exercise in 24 yr old women significantly increased plasma concentrations of bGH-L. This increase was associated with forms recovered in the > 60 kDa fraction.
- Nindl et al. [13]. The hypothesis that bGH-L concentrations in plasmas from 80 physically fit men and women of the U.S. Military Academy would be related to a) their bone mineral densities; b) other bone biomarkers; c) IGF-1 or its binding proteins and d) acid labile subunit measures was not supported. Concentrations of iGH in plasma samples from female cadets were significantly greater than those of the male cadets.
- Thomas et al. [14]. bGH-L in plasma samples from 9 men [BMI = 23], taken during and after an acute bout of heavy resistance exercise, were significantly higher relative to those taken from obese [BMI = 36] men. Yet neither groups showed significant increases in bGH-L after exercise stress. Plasma concentrations of iGH were not different between the two groups (Fig. 3).
- Gosselink et al. (unpublished). Concentrations of plasma bGH-L during and after treadmill exercise in 5 adult (28 yr) males increased an average of 2.5 fold during the 15 min exercise period, but gradually fell to pre-exercise concentrations 45 min later. Conversely, iGH concentrations were no different from pre-exercise levels during the exercise period, but gradually rose 4 × by 45 min post exercise (Fig. 3)
- Gordon et al. [15]. Comparison of bGH-L plasma levels from 24 vs 62 yr old female volunteers, after acute aerobic cycle exercise, were not different. However, with an additional acute resistance exercise bout plasma concentrations of bGH-L from the younger group were significantly higher than those in the older group. These higher bGH-L concentrations were associated with molecular forms of apparent mass 30–55 kDa (i.e. dimer range).
- Kraemer et al. [16]. This trial, done in free-living 81 yr old individuals, failed to reveal differences in either plasma bGH-L or iGH that could be correlated with either fitness or physical performance. One half of the test subjects ($n = 21$) had plasma bGH-L concentrations that were at the limit of detectability, i.e. were essentially zero. However bGH-L was readily detected in samples from the 20 other test subjects. Statistical hierarchical regression models predicted that other biomarkers (GHRH, c-peptide, VEGF, NPY, IL-4 and T-regulatory lymphocytes) could collectively account for (or be impacted by) bGH-L presence in those 20 samples.
- McCall et al. [10]. Three trials tested the overarching hypothesis that neural afferent inputs from skeletal muscle modulated human pituitary bGH-L secretion; Collectively, these experiments provided the initial evidence in humans for a *muscle afferent-pituitary axis*. These three trials tested male subjects: (1) at complete bed rest for 17 days [17]; (2) after vibration-induced activation of muscle afferents [18] or (3) pre-, during and post 17 day spaceflight in astronauts after exercising [19]. In 2 of these studies, the exercise component was unilateral ankle plantarflexors. Collectively, the results showed that acute muscle afferent activation increased plasma bGH-L. Further, these investigators found that they could increase plasma bGH-L simply by vibrating human muscle afferents originating from the tibialis anterior (but not the soleus) muscle. The authors postulated that disruption of exercise-induced bGH-L release from the pituitary gland (such as what would be expected during chronic bed rest or spaceflight) was the result of chronically altered patterns of activity of muscle afferents. *In each of these 3 human trials, concentrations of plasma iGH were not affected by treatment*. These findings demonstrate that bGH-L release in response to exercise is dependent on loading of the musculoskeletal system.
  The variability in the exercise responses observed in bGH-L is most likely due to the many different external variables that modify the anterior pituitary's content, cellular plasticity. Such variables as age, sex, fitness levels, body composition, diet, environment, exercise modality, and training state may all contribute to variations ascribed above. Additionally, the nature of the exercise stimuli will play a major role in both responses and adaptations.

4. Section II. What rat studies tell us about bGH-L

4.1. Experimental series 1. Rat hind limb controls pituitary bGH-L

In the 1970's, studies by Ellis and Grindeland used both rat plasma and rat pituitary gland extracts to reveal the presence of a potent pituitary growth factor that was undetectable by antibody based methods, but was active in the tibial line bioassay. The growth factor was termed bioactive GH to distinguish it from the form readily detected by immunoassay (i.e. the native 22 kDa GH); GH antisera failed to neutralize the growth response and in this respect, as pointed out by Blumenthal [20], was similar to the behavior of nonsuppressible insulin like activity (NSILA), an activity ultimately attributed to IGF-1 and IGF-2 peptides. Their results indicated a bGH-L potency equivalent to ~300 times that of iGH. At that time, a metabolic link also was proposed, because pretreatments of rats, e.g. insulin-induced hypoglycemia, fasting or exposure to cold reduced pituitary bGH-L content by ~65%; increased concentrations of plasma bGH-L; but had no effect on concentrations of plasma or pituitary iGH.

Later, Grindeland, Hymer and their colleagues reported major differences between iGH and bGH-L secretion in rat pituitary tissue after exposure to a low gravity environment [21]. In turn, these additional findings stimulated experiments which tested the hypothesis that proprioceptive perturbations in the rat neuromuscular system would also modulate pituitary bGH-L release. These studies led to the discovery of a novel muscle afferent-pituitary axis that regulated bGH-L secretion. Rats trained to run on a treadmill for 15 min at 27 m/min had plasma concentrations of bGH-L that were 300% higher and pituitary bGH-L concentrations that were 50% lower than controls, but iGH concentrations in these same samples were not different from controls [22]. Furthermore, stimulation of certain hind-limb nerves differentially affected bGH-L secretion. Specifically, stimulation of the peroneal or tibial nerves (which innervate fast-twitch muscle) increased plasma bGH-L up to 250% while reducing pituitary bGH-L content by 60% [23]. In contrast, cutaneous sural nerve stimulation had no effect, and soleus nerve stimulation or activation of slow-twitch muscle inhibited bGH-L

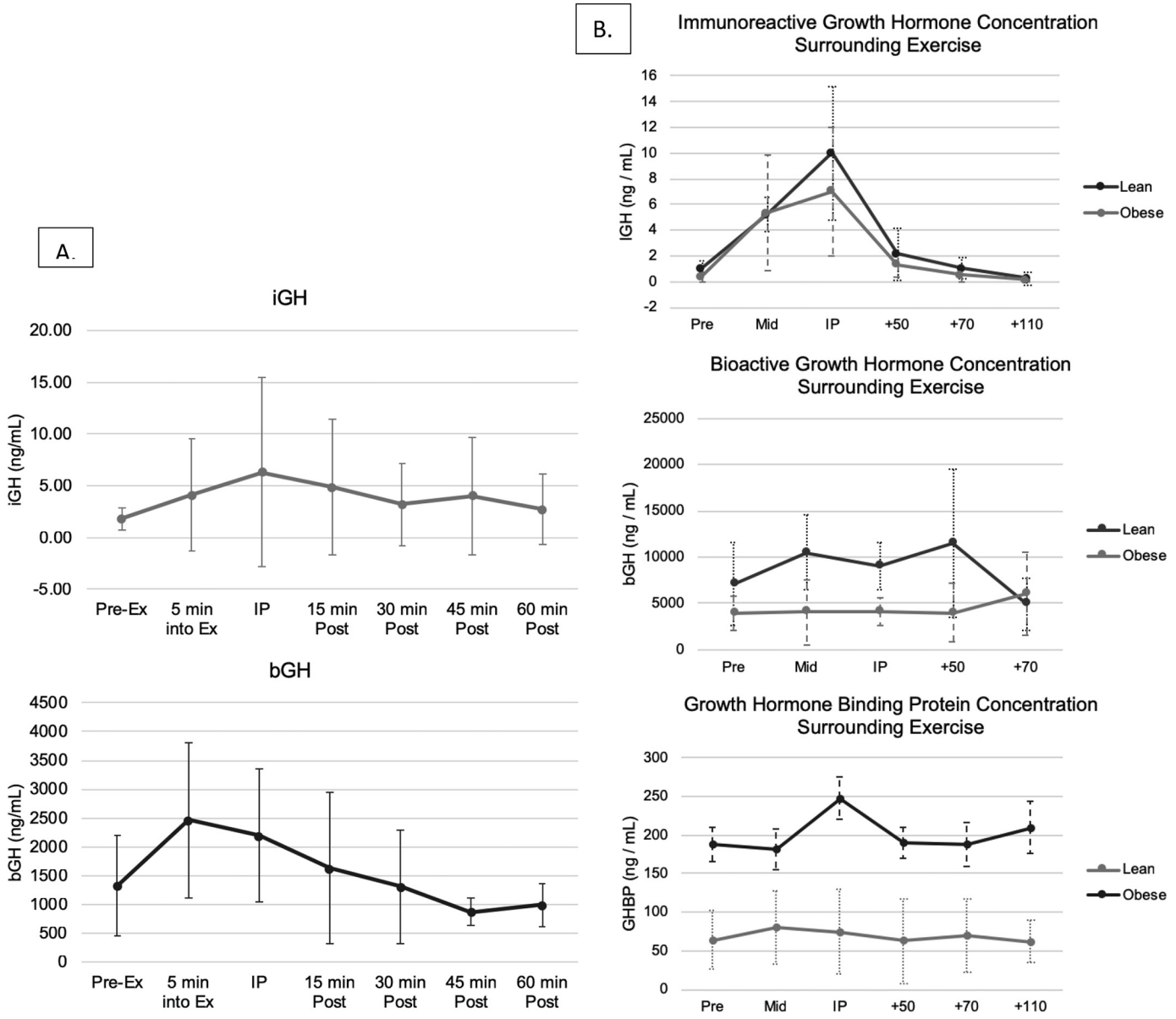


Fig. 3. Plasma responses of immunoreactive (IGH) and bioactive growth hormone (BGH-L) A. in men before and after running on a treadmill for 15 min at 70% of predicted VO2 max (Gosselink, KL, unpublished data) and B. Responses of IGH, BGH-L and GH Binding Protein in men (normal body mass and obese) before and after a resistance exercise workout (adapted from ref. 13). No significant differences were observed with exercise but men who were obese had significantly ($P \leq .05$) lower BGH-L concentrations at pre, mid-, post exercise and 50 min into recovery, GHBP was significantly lower at all of the time points for men who were obese. IGH concentrations were significantly lower for pre and immediately post-exercise time points.

secretion [10]. The stimulation patterns in these studies identified low threshold, large diameter afferent fibers as a mechanism for eliciting the bGH-L response [24].

Activation of group 1a afferents from rat fast muscle increased bGH-L release while activation of the same sensory afferent input from slow muscle decreased bGH-L output [25]. When these muscle afferents were stimulated in animals subjected to hind limb suspension for 4–8 weeks, plasma bGH-L concentrations were significantly suppressed while those of the ambulatory controls were significantly increased [26]. Together, such studies demonstrated that specific types of nervous system afferents feedback are likely to regulate bGH-L, (but not iGH), secretion. This response depends on musculoskeletal loading history.

In summary, as in humans, a majority of the pituitary bGH-L release in rats occurred in the first 15 min after treatment. Furthermore, activation of fast or slow muscle afferents had opposing effects on bGH-L release. The precise muscle pathway(s) that control pituitary bGH-L might involve well-established neuronal projections from the lumbar spinal cord to the hypothalamus via the spino-hypothalamic tract thereby leading to the release of GHRH into the portal system [27].

A second (and intriguing) pathway possibility is suggested by the collective findings of Vanhatalo [28], Liu [29] and Ju [30–32]. Combinations of neuroanatomical tracing, denervation, and immunochemical techniques demonstrated that substance P fibers directly innervate the anterior pituitary gland via the nodose (inferior cervical) ganglion; and b) that substance P, calcitonin gene related peptide and galanin positive nerve fibers are present in the anterior pituitary of humans, monkeys, dogs and rats. Their findings raise the possibility that both hormonal (hypothalamic peptides) as well as direct peptidergic neural connections to the pituitary, could theoretically regulate bGH-L secretion.

Such findings lead to the hypothesis that… *"acute metabolic stress and/or activation of specific muscle groups play an important role in*

Table 1
Concentrations of Bioactive Growth Hormone (BGH) in the Pituitary Glands of Post-Mortem Individuals at Different Ages, adapted from (7).

Age	# of Individuals	BGH	CI-95%
< 5 months	22	0.2	0.1–0.3
5–8 months	54	0.8	0.3–2.0
8–9 months	33	1.1	0.5–3.0
0–0.5 years	8	3.3	2.0–5.0
0.5–12 years	5	3.1	2.0–6.0
12–18 years	4	8.7	5.0–15.0
18–30 years	10	4.6	2.0–12.0
30–55 years	128	6.4	4.0–10.0
55+ years	278	9.9	7.0–14.0

BGH Concentration: IU $\bullet 100^{-1}$ mg dry weight.

controlling carbohydrate metabolism by mobilization of bGH-L during threats to homeostasis" [26]. They may also relate to recent studies which indicate that rhGH therapy enhances functional recovery of injured (and severed) peripheral nerves [33,34].

5. Experimental series 2: rat pituitary cell suspensions

To study bGH-L production and/or release from a somatotroph cell, one is forced to destroy the 3 dimensional architecture of the gland by harsh enzymatic digestion. Nevertheless, the superb ultrastructural study by Hopkins and Farquhar in 1973 [35] established that the morphologic and functional integrity of somatotrophs was preserved in cell suspension. To our knowledge, these investigators were the first to suggest functional heterogeneity in the somatotroph population because one half of the somatotrophs failed to incorporate ^{3}H leucine into new protein. These early experiments set the stage for literally hundreds of pituitary cell culture experiments in the ensuing years; many of which were aimed at defining mechanism of action studies of secretogogues on GH cells.

But do somatotrophs, in single cell suspension, retain their ability to produce biologically active GH in vivo? Prior to 1970, attempts to restore body growth in the hypophysectomized rat used whole gland transplants; such trials met with limited success. However, Halasz et al. [36] had shown that transplantation of the intact gland into the hypophysiotropic area (medial basal hypothalamus) of the brain resulted in partial growth; a result which prompted Weiss et al. [5] to implant dispersed rat pituitary cells into the brain ventricles of the hypophysectomized rat. Cell dose dependant weight gains of 20–60% in the implanted animals, over a 30 day post-implantation period, were reported. Tibial and femoral bone lengths were significantly longer and body composition analysis also confirmed the anabolic character of the hormone secreted from the implanted cells. That somatotrophs in such cell suspensions caused these anabolic changes was further established by the finding that implantation of 0.6×10^{6} somatotrophs, (purified to 90%), caused weight gain.

5.1. Experimental series 3: rat pituitary cell separation studies

That dichotomy in bGH-L vs iGH activity measurements might have its origins within the pituitary gland itself was first suggested by early in vitro studies (1963–1972) [1,37]. About this same time, methods were being developed to separate different types of hormone-producing cell types contained in trypsinized rat pituitary cell suspensions [38–40]. These methods were based on differences in physical characteristics of the cells, viz. their size and/or density. Density gradient centrifugation enabled somatotroph enrichment from ~35% (in initial cell suspensions) to 85–90%. Further, these studies revealed that two populations of GH cells were present in initial cell suspensions; one (light somatotrophs, also termed type I cells) had densities < 1.071 g/cm^3, while the other (heavy somatotrophs, also termed type II cells) had densities in ranges > 1.071–1.085 g/cm^3. *Each of these two fractions accounted for ~ 40% of the total somatotrophs contained in the initial cell suspension* [38]. Swedish investigators subsequently confirmed the existence of 2 subpopulations of somatotrophs in the rat pituitary [41–43]. Dense cells (> 1.065 g/mL) appeared heavily granulated with "sparse" regions of endoplasmic reticulum and Golgi. Less dense cells were sparsely granulated, but had abundant regions of endoplasmic reticulum and Golgi. *Their data also indicated that ~48% of all somatotrophs were of the dense type.*

These cell separation studies were important for two reasons: first, they unequivocally established heterogeneity of the GH producing cell population in the rat pituitary and second, set the stage for experiments to find out if the GH released from light vs heavy somatotrophs was differentially active by bioassay. The answer came in 2017 report by Grindeland et al. [44]. Their study showed that: 1) media from dense cells in culture for 12 days, when tested by tibial assay, contained 5× as much bGH-L as that from less dense cells; 2) although net production of bGH-L from dense cells was 6× more than that from less dense counterparts ($p < .001$), production of iGH was not different between the two types; 3) implantation of dense cells into rat brain ventricles of hypophysectomized recipients significantly increased body weights, tibial widths and gastrocnemius muscle weights; 4) implantation of less dense cells had no effect on tibial widths or gastrocnemius muscle weights; and finally 5) pituitary cell donors prepared from either fasted or insulin injected rats reduced subsequent in vitro secretion of bGH-L from the dense cells 2-3×, [both treatments were known to cause massive depletion of pituitary bGH-L content in vivo with concomitant increase in plasma bGH-L [1]]. (see Table 2).

Other studies show the impact of pituitary donor age on bGH-L cell function Study #1 [5]. Implantation of 2×10^{6} cells (not fractionated) from 70 day old rats promoted weight gains of 60% after 30 days post-implantation, while cells from 30 day old animals had weight gains of 30%; a result which suggested more intracellular GH in the somatotroph of the older animal-similar to the human pituitary (Table 1). Study #2 [38]. After cell separation, the percentages of type II somatotrophs prepared from 25, 65 and 120 day old male donor rats (corresponding weights 70, 250, 400 g respectively) were of the same magnitude; viz. 50 +/− 5%, but their intracellular concentrations of GH increased as the animal aged (0.14/0.25/0.36 ng GH/type II cell respectively). This result shows increased secretory granule content of GH/cell. Study #3 By flow cytometry, it could be shown that the number of cytoplasmic secretory granules in the dense, type II cell increases as the rat ages from 24 to 91 days old [44].

Table 2
Type I and Type II Somatotroph Characteristics (Means), adapted from (44).

Somatotroph Characteristic	Type I		Type II	
	IGH	BGH-L	IGH	BGH-L
Total GH *in vitro* secretion (ng per1000 somatotrophs)	654.0	334.0[a]	653.1	2,106.7[a]
	Type I		Type II	
Responses of Hypophysectomized Rats Implanted with Rat Pituitary Cells for 12 Days				
% Change Body Weight	3.5		9.9[a]	
Tibial Epiphyseal Width (μM)	171		197[a]	
Gastrocnemius Weight (mg)	563		615[a]	

[a] ($p \leq 0.05$) different from corresponding somatotroph type

5.2. Experimental series 4: somatotroph plasticity and geometry driven networks in mouse and rat pituitary

5.2.1. Plasticity and GH dichotomy

The past 20 years have seen remarkable progress in revealing details underlying the plasticity of the hypothalamic-vascular-anterior pituitary gland complex. Major advances in tissue imaging technology in vivo revealed that both the endocrine and non-endocrine cells of the anterior pituitary gland were organized into structural and functional networks. The studies of Mollard, LeTissier and their colleagues during this period clearly demonstrated that the conventional 2D view of the gland missed a higher level of organizational (and functional) properties of the entire somatotroph population. Further, their results indicated that geometry-driven changes correlated with GH cell function.

Major findings from this group indicate that: a) the GH network is sexually dimorphic; b) the GH network shows functional plasticity as it relates to the physiology of the animal; and c) GH network plasticity continues into adulthood, and although still present in the gland of a 660 day old mouse, the GH network becomes more "loose" as the animal ages [45–47]. LeTissier et al [48] argue that such results show that previously held views on mechanisms regulating the hypothalamic-pituitary system are "over-simplistic"and need to be considered in the context of network plasticity which ultimately impact the activities of circulating hormones in a healthy or disease state.

GH cells form a dynamic, reversible functional network; i.e. all GH cells, in intercrossing strands (cords) of single GH cells are connected to each other by adherens junctions. Importantly, these single cell strands also form clusters of closely apposed somatotrophs by crossing over each other to form nodes and strands [46]. The extent of strand interaction, and network cell clustering was found to be directly correlated with increased body weight and pituitary GH content of the tissue donor. Exposure to GHRH in vivo changed the local pattern of cell-cell communication. Other experiments showed that a) in the oldest mice (100 days), GH cell clustering was reversible and dynamic b) castration suppressed clustering in the lateral wings, but had no effect on clustering in the medial zone and c) GH network responses were sexually dimorphic.

Studies from other investigators also show that location of the somatotroph in the individual rat pituitary gland makes a difference in regional control of somatotroph function. The method used in these studies used slices of single glands into either 8 [49], 2 [50] or 4 [51] pieces prior to a) culture for GH assay and b) flow cytometry (to determine somatotroph percentages in each slice following enzymatic dissociation). Secretion of bGH-L (tibial assay) was 5× greater from somatotrophs located in ventral slice regions vs those in the dorsal regions. However, secretion of iGH was 1.5-2× greater in dorsal sections than that in the ventral regions [50]. A similar approach has been used to study changes in regional somatotroph function after resistance exercise training (rat ladder climbing model) and significant pattern differences were reported [52].

In summary, the technical approaches of the Mollard group have advanced our understanding of pituitary physiology. Their findings solidify the concept that localized cell coupling, in specific regions of the gland, offer a dominant mechanism for internal control of cell function in groups of GH cells within the individual gland.

5.2.2. Somatotroph heterogeneity: additional evidence

The following studies may offer additional insight into control of bGH-L secretion, but each lack direct experimental evidence.

- Type II rat somatotrophs are more responsive to GHRH-induced release of iGH in vitro than type I cells; type I cells also show greater sensitivity to octreotide. Type II somatotrophs are more sensitive to osmotic stress (volume and shape oscillations) in vivo [13,43].
- Lafont et al. [45] imaged microvascular changes in different regions of the pituitary of the GH transgenic mouse and reported a 15 fold variation in rates of blood flow, including a 7 fold range in vessel diameters (two populations of RBC velocities were observed; one at peak velocity of 100–200 um/s and the other at 500–600 um/s). Changes in local blood flow and oxygen partial pressure were coincident with activation of GH pulses. Importantly, clearance of two different fluorescent markers (4 kDa and 20 kDa) released from GH cells into blood vessels of the live animal was also different; 4 kDa clearance was rapid, but clearance of the 20 kDa marker (approximating the size of native GH) from the perivascular space was much slower. We speculate that when the GH secretion granule undergoes exocytosis, modification(s) to aggregated storage forms during clearing from the perivascular space might account for the kinetics of bGH-L vs iGH release during a 15 min exercise period (Section I).
- Three dimensional imaging techniques show that 30% of the somatotrophs are entirely isolated from perivascular spaces while 70% are next to capillary endothelium. Cytoplasmic distributions of secretory granules are either uniform (in the former case) or asymmetric (in the later), since granules accumulate in cytoplasmic areas next to the vessel [52]. The authors point out that their results were unexpected since *"..it is widely accepted that a large proportion of the endocrine cells physically interact with capillary blood vessels"*. Because GH content/type II somatotroph increases with age of the animal (discussed earlier), it would be interesting to find out if the type II cells predominate in the pool removed from blood vessels, thus becoming non-functional.
- The vascular biology of the pituitary gland is complex. Two of three major types of capillaries (fenestrated and sinusoidal) are present in the anterior pituitary [53]. While the fenestrated capillaries have pores with a diaphragm that permit passage of solutes with varying permeability, the sinusoidal pituitary endothelium has gaps between endothelial cells which provide passage for large solutes (e.g. plasma proteins). Maintenance of such pituitary vasculature is reported to be regulated by hypothalamic releasing hormones via VEGF signaling. The reader may recall that one of the 6 plasma biomarkers found to account for differences in concentrations of plasma bGH-L in 81 year old human subjects was VEGF (Section I).
- Substance P may be a part of a regulatory network for hormone secretion from the anterior pituitary gland of several species, including the rat and human. Substance P fiber tracts are found in the caudal, central regions of the rat gland, frequently at the dorsal-ventral area, in close proximity to gland cells. These morphologies prompted Ju et al. [54] to speculate that the gland *"can be regulated by direct neural factors, as well as humoral factors"*. Interestingly, ~23% of rat pituitary somatotrophs also are immune-positive for substance P [55]. These observations are relevant to peripheral regulation of regional control of bGH-L in the rat pituitary gland (i.e. muscle-nerve afferent pathway of bGH-L control; Section II).
- The folliculo-stellate (FS) cell type is estimated to make up 5–10% of the cells in the anterior pituitary. These cells transmit information via a complex interconnecting system of follicles joined, in part, as a network by gap junctions [56]. The system is described as one in which the hormone producing cells (cords) are arranged …*"like a bunch of grapes, and the FS cells make networks in the cell cords to connect each grape"*. FS cells play important roles in inter-pituitary communication (e.g. Ca flux regulation); however essentially nothing is known about their participation in bGH-L processing and/or secretion. The pioneering ultrastructural studies of Farquhar [35] described a role for FS cells in disposal of the whole cell via phagocytosis; (most frequently in the well- granulated somatotroph). Farquhar described this process as… "*the way an octopus pulls its prey by way of its tentacles*". Phagocytosis by FS cells may play an important role in maintaining tissue homeostasis by regulating intracellular bGH-L concentrations in a subpopulation of well granulated somatotrophs located away from blood vessels.

5.2.3. Summary of section II

Somatotrophs contained in single cell suspensions of rat pituitary glands secrete bGH-L as judged by anabolic responses in the host upon implantation into the hypophysectomized rat. Cell separation studies document functional heterogeneity of somatotrophs producing bGH-L; i.e. ~ 40% of GH cells are high bGH-L producers, are dense and laden with 300 nm secretion granules. The remaining ~40% are essentially devoid of granules. Implantation of dense somatotrophs predominate in promoting positive anabolic responses in bone and muscle of the host animal. Somatotroph subpopulations appear to be anatomically and functionally organized, and are under coordinated, coupled control in specific regional areas of the gland.

6. Section III: bGH-L heterogenity, molecular variants and secretory granules

A comprehensive review of GH variants, their structures and physiological activities is beyond the scope of this review. However, in this section we offer some answers to the question: What are the molecular form(s) of GH that possess bGH-L activity? Attempts to purify GH variants (between 1975 and 2000) were directed at understanding their physiological effects. A review by U. J. Lewis in 1992 entitled *"GH: What is it and what does it do?"]* makes the point. The abstract is provocative and relevant …. *"The evidence is now irrefutable that growth hormone (GH), long thought to be a single substance, is actually a mixture of several different forms. These multiple forms must be a consideration in any physiologic study if an accurate evaluation of the actions of GH is to be made" [9]*. Fragmentation of the native 22 kDa hormone into two peptides [hGH 1–43] and [hGH 44–191] affects their physiology; the shorter fragment has insulin potentiating activity while the larger has anti-insulin activities, thereby implying that the native molecule acts as a prohormone. If GH has so many metabolic activities, is their mechanism of action via a common receptor? Lewis addresses this point in a 1996 report: "*currently it is believed that all of these actions are mediated through the cloned GH receptor, but this is not proven" [57]*.

6.1. Native (recombinant) GH

By 1985 the National Pituitary Agency had shut down production of hGH extracted from human pituitary glands because of JC virus contamination. About that same time pharmaceutical firms switched to use of recombinant DNA technology for hGH production. In spite of high assay variability, the rat weight gain bioassay for GH (first described in 1938) continued to be used at that time in "production control" for manufacture and validation of the recombinant hormone [58]. As emphasized in the Roswall report, the relative potency of a single lot of rHGH product (in 23 replicate weight gain assays) varied > 50%. Nevertheless the authors made an important point: .."*demonstration of purity and identity of rHGH monomer is not sufficient to prove biological activity, because all of the chemical and physical aspects contributing to bioactivity are not known*". [We note that assay variability of hGH is common; e.g. a "classic" paper, published in 2007, describes results of a trial in which a single rhGH sample was measured by immunoassay in 104 clinical centers in the UK and results varied 2–3 fold!] [59].

The merit of Roswall's cautionary statement had been shown in an extensive collaborative study done 4 yrs. earlier [60]. It was designed to compare results of bGH-L assays (tibia assays vs a number of in vitro cell based assays) of two rhGH preparations from two different manufacturers. Investigators from 10 independent national control laboratories, located in 8 countries, tested activities on coded (blinded) samples. Prior to testing, some of these preparations were also subjected to limited proteolysis (incubation 72 h, 4C and 0.1 μg/mg subtilisin), deamidatization or dimerization. Assays were: tibia tests [30 independent bioassays]; cell based receptor assays (Nb2, 3 T3, liver, IM-9 lymphocyte); immunoassays; reverse phase HPLC and finally size exclusion chromatography. Three results from this study are particularly relevant: first, limited proteolysis increased tibia widths in all 10 assays (x = ~50%) relative to those in the initial, untreated test sample; second, limited proteolysis decreased activities in all cell based or immunoassay tests; third activities of de-amidated and dimerized samples were not increased in the tibia test after proteolysis.

The main conclusion from this impressive collaborative study presents a conundrum; which bGH-L bioassay should one use? Bristow and Jeffcoate [60]suggest that the answer depends upon the nature of the study and questions being asked, e.g. are they physical-chemical or physiological in character? The focus of experiments summarized in sections I and II is obviously physiological. These data show that the tibial assay detects the native form (represented by rhGH) and is the major bGH-L form in the pituitary and plasma. However, based on other data in sections I and II, it cannot fully account for bGH-L activity.

6.2. 2-chain growth hormone

Enzymatic cleavage between residues threonine-142 and tyrosine-143 of the hGH molecule produces a two chain variant which consists of an amino-terminal fragment linked by a disulfide bond to a smaller carboxyl terminal fragment that hold the two chains together. Such proteolytic cleavage may occur during secretion via action of a membrane bound protease [58]. When purified by chromatography, this clipped form is "super-potent" (i.e. activated) when tested in the rat weight gain bioassay (relative potency = 1.78). However, this clipped hGH has degraded activity in all cell-based bioassays. Schepper et al. [61] offered strong support for the physiological activation theory by showing that preparations of liver (but not microsomal) plasmalemma enriched preparations contained a serine protease that cleaved the large disulfide loop of hGH, thereby accounting for enhanced biological activity.

6.3. Oligomeric forms of GH in human plasma

The distribution of GH isoforms in human serum, measured by isoform-specific assays after a 15–30 min secretory pulse, are shown in Fig. 2. How such data might relate to questions of bGH-L in human plasma is not only complicated, but must be interpreted within the cautionary requirements set forth by Lewis described previously. Two studies from our laboratories reveal that bGH-L activities of some of these isoform classes are associated with molecular complexes having an apparent mass > 30 kDa. For example, plasma samples from women who had undergone chronic resistance exercise (24 wk), when separated by gel chromatography, contained aggregates with higher concentrations of bGH-L relative to those of untrained individuals. This association suggested a possible mechanism for somatogenic adaptation after exercise training [12]. An increase in circulating GH oligomers was also found in a follow-on study comparing aerobic vs resistance exercise in both 24 and 60 yr old women [62]. This increase was coincident with decreased iGH concentrations in the exercise groups.

6.4. Is aggregated GH another form of bGH-L? (the case for GHBP)

Extensive studies by Bauman showed that ~50% of the 22 kDa GH was bound to a high affinity growth hormone binding protein (GHBP). This protein, first described in 1986, is the soluble version of the extracellular domain of the GH receptor in humans. Amino acid sequence identity was shown between the extracellular domain and purified GHBP shortly thereafter. GHBP is generated via shedding of the GHR ectodomain by tumor necrosis factor-a-converting enzyme (TACE).

Two recent reviews document the importance of the GHR in health and disease. For example, Brooks and Waters [63] point out that growth hormone receptors are present in virtually every cell of the body. They emphasize two disparate kinds of activity for GH; viz. 1) action via signaling mediators/cytokines and 2) modulation via its classic action on regulation of IGF-1 production. Curiously, dysregulation of GH

metabolism in individuals > 175 cm tall is correlated with increased incidence of breast, prostate and colon cancer [64]. The reader is also referred to a review by Schilbach and Bidlingmaier for a current summary of GHBP and its physiology in health and disease states [65].

Does GHBP by itself have bGH-L activity? Investigators at Genentech, Inc. addressed this important question in a series of complex rat growth studies in which preparations of a) GHBP alone, or b) GHBP in combination with rhGH, were injected daily into hypophysectomized rats and evaluated 8 days later for changes in weight gain, bone growth, serum hGH and IGF—*I. major* findings were that a) GHBP, by itself, had no effect on body weight gain, but "considerably" enhanced the growth-promoting effect of rhGH (~3×), and b) GHBP prolonged the presence of rhGH in the plasma. The enhancement effect was also noted in liver and kidney weights [66].

This important work would seem to support the idea that GH monomer, bound to GHBP in plasma, qualifies as yet another bGH-L isoform distinct from a disulfide linked aggregate. A molecular complex consisting of hGH and GHBP in a plasma sample would be expected to elute from a size exclusion chromatography column as an oligomer (> 30 kDa). A discussion of how the experiments by Clark et al. [66] relate to the normal function of GHBP, as pointed out by Clark himself, "can be debated". However, the concept espoused by Veldhuis et al. [67] that…… "*in vivo dynamics of GH secretion, trapping, and clearance from the circulation offer a variety of regulatory loci at which the time structure of free, bound, and total GH delivery to target tissues can be controlled physiologically*" is commonly reported and can be invoked as physiological probability.

6.5. Tibial peptide

A peptide, isolated from 6 batches of postmortem human pituitary glands (2–10 glands/batch), is active in the tibia assay[68]. The peptide has an isoelectric point of 5.1, a molecular weight of 3374 (determined by mass spectrometry), an amino acid composition high in glycine and is undetectable by rhGH immunoassay. The primary isolation tool (free flow electrophoresis), allowed continuous processing in order to obtain sufficient material for final purification by anion exchange chromatography. ~50% of the total bGH-L activity in the initial extract was associated with material of high electrophoretic mobility (anodal) and was devoid of iGH activity. Although the N terminus was blocked, limited amino acid sequence data indicated that many of the residues were nonpolar and bore some resemblance to the c-peptide of proinsulin. The internal sequence of this peptide (residues 9–25) is ASPVV-VGGGASLPEFGY. The peptide is not a fragment of the GH molecule. In order to differentiate it from rhGH, the peptide was named tibial peptide.

Gosselink and Grindeland generated a synthetic form of the tibial peptide (Synpep Corp) in order to determine its' activity in the tibia bioassay. In each of 2 assays, three doses of synthetic peptide (5, 15 and 45 μg, total dose) were injected i.p. in a total volume of 2 mL over a 4 day injection period (0.5 mL/day; $n = 5$ rats/dose). Relative to saline injected controls, the peptide was found to promote bone growth in both assays. The tibial widening responses were largely dependent upon dose, and an overall main effect was highly significant ($p < .0001$) [Gosselink and Grindeland, unpublished].

To determine if the tibial peptide was present in plasma, Hymer et al. [68] used the identical isolation scheme described for human pituitary tissue on three batches (200 mL/batch) of fresh, frozen cryopoor human plasma (15 donors/pool). The protein separation profiles between the two tissue sources were identical. The plasma peptide was active in the tibia test.

7. Tibial peptide, secretory granules, functional amyloid and the regulated pathway

Somatotrophs are professional secretory cells and as such package their cargo in dense core secretory vesicles via the regulated secretion pathway. Much is known of general mechanisms that such cells use to sort, store and transport protein product to the cell membrane for exocytosis. Of necessity, these mechanisms are largely revealed using cell culture methods and are beyond the scope of this review.

The presence of tibial peptide in both human pituitary tissue and plasma raises a number of questions concerning its relationship(s) to native forms of bGH-L, sub-cellular processing site(s) and physiological importance. These issues, considered in the broader context of intracellular processing of the native 22 kDa molecule, are considered in this section.

Purified preparations of 300 nm rat pituitary cytoplasmic granules, on injection into the hypophysectomized rat, are active in the tibial assay. They account for ~75% of the total bGH-L activity in the initial homogenate [74]. In a granule, the hormone, bound to Zn(II) ion, exists in aggregated form at high concentration (~4 mM) with two Zn(II) ions cooperatively associated per GH dimer [75]. Each granule is estimated to contain 5000–10,000 molecules[76]. The dense core of the GH granule consists of large, crystal-like reversible aggregates which solubilize upon exocytosis [69,70]. Some GH granules contain cytochrome C, cytochrome oxidase and ATP [79,80,77]. > 35 years ago, Lorenson and her colleagues showed that Zn-ATP interactions functioned to modulate GH release from the bovine GH granule in vitro [69].

On electrophoresis in non-reducing SDS gels, rat pituitary extracts contain a wide range of S—S linked GH variants (14–88 kDa MW) [71]. Electro-elution of protein from different regions of such gels, followed by their chemical reduction, apparently uncovers epitopes hidden in the aggregate, thereby increasing iGH activity up to 6×. Forms > 44 kDa are found exclusively in extracts prepared from dense, highly granulated, purified type II- bGH-L producing- somatotrophs (pentamers). Extracts from the less dense, less granulated, type I somatotrophs contain a single dominant 22 kDa peak and a minor 44 kDa species (dimer). Chemical reduction of culture media from type II, but not type I, somatotrophs increases immunoreactivity (5× vs 1.3× respectively). This important result confirmed maintenance of granule heterogeneity within the somatotroph in cell culture. *Since GH released from the type II somatotroph, relative to type I cells, is most active in both* in vitro *(cell culture) and* in vivo *(hollow fiber implant) bGH-L tests (section II), our results [71] [44], support the contention that bGH-L activity is associated with disulfide linked aggregates residing in granules of the type II somatotroph.*

The more recent reports by Maji et al. [84,85] showing that GH is stored as a functional amyloid within the granule was a major advance. Amyloids are defined by their highly organized cross B-sheet regions in protein or peptide aggregates. The cross B sheet represents a single structural epitope in which individual strands of each sheet run in perpendicular to the fibril axis while B-sheets are parallel to the fibril axis. These highly organized amyloid fibrils are composed of thousands of copies of B strands composed of peptide/protein. The B strands can be stacked in parallel or antiparallel orientation. In many proteins the amyloid state is thermodynamically stable at high concentration and not energetically favorable at lower protein/peptide concentration [72].

A battery of biophysical techniques used by the Maji group predict that residues 72–82 of the 191 rHGH monomer have a high aggregation propensity and that 4 fibrillation segments, each of ~6–10 residues, are B aggregation "hot spots". In vitro studies show that only Zn(II) ion, as the specific helper, allows fibrillation [73]. These aggregates bind amyloid specific dye (Congo Red) which yields birefringence-a hallmark signature of an amyloid protein. Further, aggregates formed in the presence of Zn showed binding to Thioflavin dye and cross B sheet structure as revealed by FTIR and reflections from X-ray diffraction of fibrils. These results not only confirm the amyloid nature of the hormone in the granule, but also show that most of the molecule was able to maintain its globular fold, even after fibril formation. The amyloid

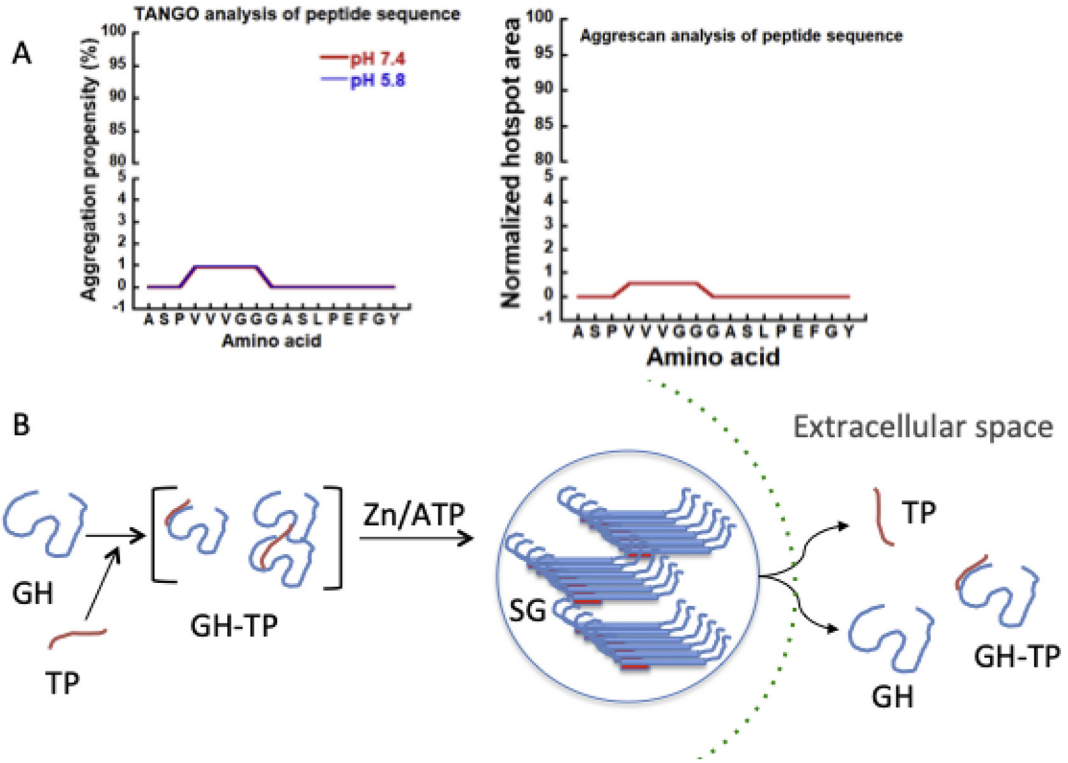


Fig. 4. A. TANGO and AGGRESCAN algorithm analyses show only a slight tendency for tibial peptide to form amyloid or aggregate. B. Schematic showing how the tibial peptide (TP) might interact with growth hormone (GH) and Zn in a pituitary secretory granule (SG). (from Maji, S.K. unpublished) (color to be used for this figure).

configuration not only may ensure efficient release of 22 kDa GH from the amyloid depot, but also protect the GH from enzymatic degradation, high temperature and large pH ranges.

How might tibial peptide and the GH molecule(s) (in their amyloid configuration) interact within the secretion granule? Analyses by Tango and Aggrescan algorithms indicate only a slight tendency for the tibial peptide to form amyloid or aggregate. We suggest that tibial peptide might play a connecting role with GH molecules and Zn(II) in their amyloid configuration (Fig. 4).

GH is known to form amyloid-like fibrils in some human pituitary tumors [74]. If and how these may differ from amyloid in the pituitary secretory granule is unknown.

8. Tibial peptide, biomolecular condensates and the constitutive pathway

8.1. Constitutive pathway

The mouse pituitary cell line AtT-20 has two pathways of protein secretion; 1) the regulated pathway, used for release of cargo stored in dense core granules upon trigger from an external signal and 2) proteins in secretory vesicles that release contents by exocytosis without external stimulus. It is commonly held that most cells have this default pathway of secretion. Innovative experiments revealed that cytoskeletal elements in AtT-20 cells transported organelles of both pathways non-selectively to the cell tips for exocytosis [89]. Proteins in the constitutive pathway fail to be sorted properly during transit from the ER to the golgi network, but are instead transported in small constitutive vesicles to different cytosolic regions. Studies by Levine et al. [75] indicate that the sorting efficiency of this process, in signal-containing proteins, vary widely depending upon signal, substrate and cell type (e.g. from > 95% to < 60%). These authors suggest that at least 2% of total synthesized cellular material has access to the constitutive pathway.

Similarly, and somewhat surprisingly, Chakrabarti et al. [76] estimate that 1/3 of all cellular proteins need to be targeted to the secretory pathway. A processing inefficiency of 0.1% would result in a "*substantial and constant source of non-translocated proteins, most of which require disposal*".

To the best of our knowledge, there are no data to support the idea that any molecular form of bGH-L is processed and secreted by the constitutive pathway. However, examples of experimental induction of the misrouting of iGH flow via the constitutive pathway include: hypothalamo-pituitary disconnection in sheep in vivo [77]; site directed mutagenesis of rHGH [to generate a form lacking the first 6 N-terminal amino acids] followed by gene transfer to the rat submandibular gland [78]; and use of the Cpe^{fat} mouse [which carries a mutation in the carboxypeptidase E gene, thereby resulting in removal of basic residues at the C-terminus and prohormone processing failure] [79]. In each of these three studies the default pathway leads to increased plasma iGH; but in all cases such cells fail to respond to stimulatory agent.

Exosomes and small vesicles are nano-sized particles that contain unique molecular compositions of protein, lipid and nucleic acid. They offer a mechanism of intercellular communication and tissue cross-talk. For example, after cycling exercise in healthy humans, over 300 protein classes were found to circulate in plasma packaged as extracellular vesicles [80]. It is tempting to speculate that tibial peptide also could be released from the human pituitary somatotroph in the form of an exosome/extracellular vesicle.

Biomolecular condensates. In the last decade there has been an explosion of scientific papers describing liquid-liquid phase separation (LLPS) processes that lead to formation of membrane-less organelles, existing as liquid droplets, in the cytoplasm. The LLPS process results from de-mixing in a homogenous solution and rearrangement of distinct regions of space within the droplet to form condensate. The net result is a high, local density of protein. It is thought that membrane-less organelles offer the possibility of dynamic molecular interchange with neighboring ions/molecules (metabolites). Wheeler and Hyman [81]

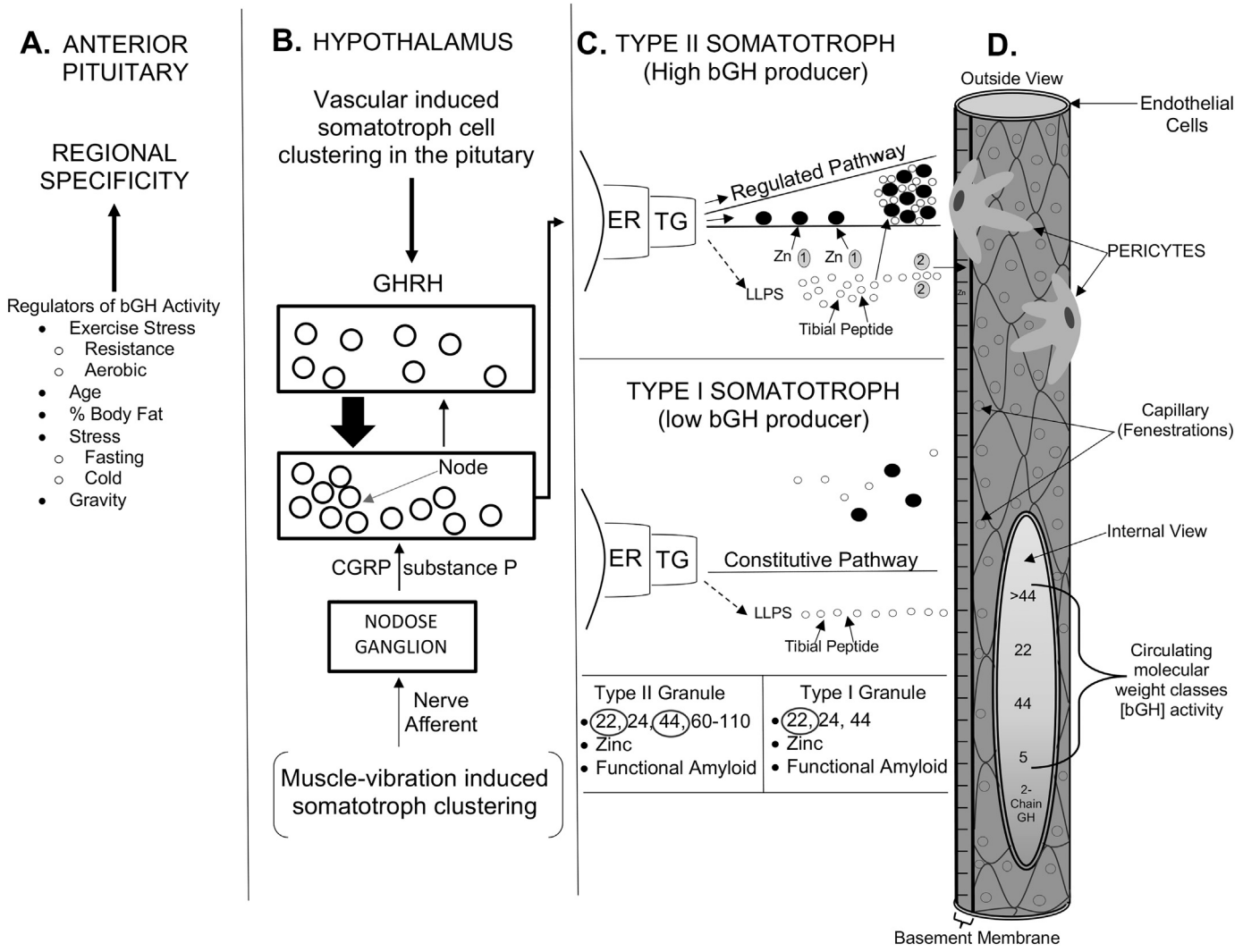


Fig. 5. Model depicting essential points underlying physiological regulatory element for bioactive growth hormone (bGH-L) production and release from the pituitary. In response to signal from a) the hypothalamus and/or b) neural afferents from the ankle flexors (panel B), some growth hormone (GH) cells (Type II) are induced to cluster into nodes within the GH cell network. Clustering induces Type II somatotrophs to produce, store (panel C) and release bGH-L (panel D) bGH-L forms into the perivascular space for further processing. Tibial peptide may serve as 1) a connecting function (see Fig. 4) or 2) is speculated as in this figure to be contained in ~5 kD bGH-L forms via a constitutive-like pathway (pathway 2). Because Zn has been shown to enhance fusion of the liquid droplet with different molecular components in other~~ biological systems (Du and Chen, Science, 2018, 361,704), we speculate that this cation also could play a similar role in "super-potent" forms of bGH-L (also depicted in regulated pathway). ER = endoplasmic reticulum, TG = trans-Golgi.

suggest that cells have "a significant" capacity to modulate these condensates and hypothesize that they offer a common mechanism to control and compartmentalize their biochemistry. We hypothesize that although membrane-bound organelles (such as a somatotroph secretion granule) are likely to be physiologically stable, a condensate is likely to be labile and sensitive to the cytoplasmic environment. Many reports indicate that the process is sensitive to solution conditions similar to those considered to be typical biological stress factors, e.g. temperature, pH, ionic strength, redox potential, and osmotic pressure.

What drives the formation of a biomolecular condensate? Intrinsically disordered regions in a protein, serving as a scaffold for multiple distinct short linear amino acid motifs, is essential. The amino acid sequence in these peptide motifs are often repetitive, typically < 10 amino acids, and are also termed low-complexity domains. In vitro experiments consistently show that phase transitions from liquid to gel to fibrillar aggregate (and reversibility of these transitions) is likely to occur in these sub-compartments [82–84]. The number of these low-complexity domains in biological systems has been estimated to be ~100,000; and within these short domains the number of potential post-translational modification sites is estimated to be ~ 1 million [85]! Finally, Zn(II) has been found to increase liquid phase condensate formation in other biological systems [86], somewhat similar to the "helper" role this divalent cation plays in GH storage in the secretion granule [73].

How LLPS behavior is encoded in an amino acid, as well as in the primary sequence of the low complexity region, is not well understood, especially as it relates to a specific biological activity. Pak et al. [87] argue that the driving force for LLPS depends upon the overall amino acid composition and not its precise sequence. Martin and Mittag [88] further classify these regions into subcategories based on frequency of specific amino acids they contain. Examples of region subcategories include those which contain a) charged amino acids that will de-mix when paired with oppositely charged amino acids; b) high fractions of polar amino acids, e.g. ser, glu, asp, and gly; and c) repetitive, homotypic sequences of polar amino acids in the presence of aromatic and hydrophobic moieties.

Given such characteristics, could tibial peptide function as a biomolecular condensate and scaffold in a liquid cytoplasmic droplet? The

experiments of Miao et al. [89], investigating sequence and structural determinants for self- aggregation of human elastin, offer insight. The assembly of human tropoelastin monomer into elastin polymer is accomplished via 34 exons in the tropoelastin gene which code for either hydrophobic or cross linking domains. Tandem repeat sequences contain GVGGVP that include an internal 3-fold repeat of PGVGGV. When tested by itself, the synthetic polypeptide will coacervate. Based on a) the amino acid character, b) the molecular features of a low complexity region and c) sequence similarity of tropoelastin domain repeats to tibial peptide (ASPVVVGGGASLPEFGY), we speculate that tibial peptide should be able to form as a biomolecular condensate.

Our working model (Fig. 5) is based on data reviewed in this paper. It depicts events within the pituitary that influence content and release of bGH-L.

9. Summary/conclusions

Prior to the development of specific, antibody- based methods to measure GH in the 1980's, bioassays such as weight gain or widening of the tibial epiphysis in the young hypophysectomized rat were routine. In 1978 Ellis, Vodian and Grindeland [1] first reported the existence of a potent pituitary growth factor that escaped detection by immunoassay, but had GH activity in the tibial assay. This factor was estimated to have ~300× the concentration of that measured by immunoassay. Termed bio-assayable GH (bGH), this growth factor was deemed physiologically relevant because it showed differential regulation after physiological challenge to the animal [10]. In this review we consider more recent information on bGH (which we rename bGH-L) to offer a framework for future studies, especially as they relate to human exercise and aging. We also discuss possible sites of bGH production within the pituitary gland-especially in relation to heterogeneity and plasticity of GH-producing cells. Lastly, we review information concerning storage forms of GH in the secretory granule, GH functional amyloids and their relationship to a human pituitary peptide that has bGH activity, but with unique amino acid sequence.

It is likely that the pituitary gland contains many growth factors (peptides) that have tibial plate activity, some of which are related to 22 kDa GH, but others which are not. None of these have been purified to the point of yielding complete primary sequence, except for rhGH.

The proposed interrelationships between phase separated condensates containing tibial peptide and the GH secretory granule is based on recent studies which show that synapsin, a major component of synaptic vesicles, will form scaffolds via LLPS where they play a role in neurotransmission at the axon terminal by maintaining the vesicles in tight clusters as well as permitting exchange of molecular contents with their surroundings. When exposed to calcium/calcium-dependent protein kinase II and ATP, the liquid droplets dissolve, thereby promoting release of neuro transmitters via exocytosis (Milovanovic et al. *Science*. 2018 Aug 10;361(6402):604–607; Boczek & Alberti Science. 2018 Aug 10;361(6402):548–549).

The type I somatotroph contains many fewer secretory granules (panel C). Any tibial peptide produced in this cell sub-population would be expected to release the ~5 kDa form of bGH-L into the pericapillary space via a constitutive-like pathway. Molecular heterogeneity of GH forms contained in the granules of the two sub-types f GH cells, shown in the study by Farringdon and Hymer [71] is also shown in panel C. Predominant forms in each granule type are circled.

An array of possible GH molecular weight classes is shown in the capillary (panel D). These forms are all know to possess bGH-L activity. Finally, the concept that events and processes controlling bGH-L (panels B, C, and D) are likely to take place within specific regions of the single anterior pituitary gland is suggested in panel A. These processes are dynamic and controlled in part by physiological/environmental factors listed in panel A.

Declaration of Competing Interest

None.

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