Understanding peptide biology: The discovery and characterization of the novel hormone, neuronostatin☆

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ABSTRACT

The Human Genome Project provided the opportunity to use bioinformatic approaches to discover novel, endogenous hormones. Using this approach we have identified two novel peptide hormones and review here our strategy for the identification and characterization of the hormone, neuronostatin. We describe in this mini-review our strategy for determining neuronostatin's actions in brain, heart and pancreas. More importantly, we detail our deductive reasoning strategy for the identification of a neuronostatin receptor and our progress in establishing the physiological relevance of the peptide.

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Introduction

The pathway from a peptide's discovery to final elucidation of its physiologically relevant actions and pathophysiologic significance is not scripted and can take many turns. Once a novel peptide is isolated and structurally characterized, the journey begins with identification of pharmacologic activity, as well as sites of production and action. Necessarily the peptide's mechanisms of action must be elucidated, including matching it to a membrane receptor and to the downstream, post-receptor signaling cascades it activates. With peptide structure and cognate receptor in hand agonists and antagonists can be developed with potential therapeutic value.

Our journey, one that began at the same time as the initial launch of the journal Peptides, has evolved with time to include many technical developments that allow us to seek the physiological relevance of numerous peptides. This mini-review will highlight our experiences as a template for the roadmap along journeys that continue today with the recent discovery of novel endogenous peptides and our attempts to understand their function.

We have maintained a focus primarily on the rat as our animal model because of its size and our ability to conduct cardiovascular and endocrine manipulations with frequent handling and minimal stress. We recognize that the mouse represents an important animal model due to the many genetically engineered approaches available, but we have developed the ability to transiently compromise peptide and receptor production in the rat with the added benefit of the reversibility of most of our approaches (Table 1). To be sure there are advantages to both animal models and the future promises to bring more transgenic approaches into the rat model. What we describe here is an example of the approach we have used to assign function to and determine physiologic relevance of newly discovered peptides using the rat as a model system.

The roadmap: from peptide discovery to receptor matching and evidence for physiologic relevance

In 2005, a collaboration began between the laboratory of Willis K. Samson at Saint Louis University and that of Aaron J.W. Hsueh at

Table 1
Non-transgenic approaches to the understanding of a peptide's physiological relevance: compromise of function and production.

	Target	References
Compromise of function		
Passive immunoneutralization	Corticotropin releasing factor	[16]
	Oxytocin	[18]
	Neuropeptide B	[21]
	Obestatin	[7]
Small interfering RNAs (siRNAs)	Neuronostatin receptor (GPR107)	[13]
	C-peptide receptor (GPR146)	[11]
Cytotoxin-based cell targeting	Oxytocin receptors	[19] [22,23]
	Natriuretic Peptide receptors	[17]
Compromise of production		
Antisense oligonucleotides	Adrenomedullin	[20]
	Nesfatin-1	[13]
Ribozymes	Adrenomedullin	[24,25] [26]
Small interfering RNAs (siRNAs)	Neuropeptide W	[27]
	Phoenixin	[11]

Stanford University. This collaboration brought together our skills in evaluating the in vivo function of peptides with the Hsueh laboratory whose expertise was based in novel bioinformatic-based strategies for the identification of previously unidentified, endogenous peptides. This represented the beginning of a longstanding collaboration that continues today. Using a computer program developed in the Stanford laboratory, Dr. Hsueh searched for cleavage sites in known prepro-hormone sequences that might suggest the production of additional, biologically active peptides from the same gene product. He was interested particularly in prepro-hormone sequences known to encode hormones (i.e. peptides) that activated G protein-coupled receptors included in the Human Plasma Membrane Receptome Data Base (www.receptome.org). Numerous potential cleavage sites in known prepro-hormones were initially identified and the list of potential candidates of interest further screened for evolutionarily conserved sequences [7]. One of the sequences identified resided in the pro-somatostatin protein, a predicted sequence we would later name neuronostatin. At that point it was incumbent upon the collaborative team to purify the predicted peptide from animal tissues, verify the predicted sequence and move forward with the characterization of the peptide's sites of production and action. Since neuronostatin was predicted to be encoded in the pro-somatostatin prohormone, identifying sites of production was not difficult based upon the existing literature [3].

A polyclonal antibody to neuronostatin was raised in rabbits and was used in co-localization of neuronostatin and somatostatin and developed a radioimmunoassay (RIA) and an enzyme-linked assay (ELISA). The antibody also facilitated the immunoprecipitation of peptide from rat hypothalamus and spleen for subsequent purification and MALD-TOF verification. We determined the immunoprecipitation-purified neuronostatin to be the appropriate molecular weight for the predicted 13 amino acid peptide sequence with, importantly, C-terminal amidation, which we would later determine was essential for biologic activity. From that point on, all peptides employed by our group were the 13 amino acid, C-terminally amidated form of neuronostatin. As predicted, both neuronostatin and somatostatin immunoreactivities colocalized in a variety of cell types, including hypothalamic neurons, pancreatic

delta cells, parietal cells of the oxyntic mucosa and villi of the small intestine [1,7].

Then began the task of determining neuronostatin's biologic actions. Based upon our knowledge of sites of production, we predicted actions in hypothalamus, pituitary gland, pancreas and gastrointestinal tract. Because somatostatin is present in cardiac afferents, we also predicted physiological actions in the heart. Initially, we took a whole animal approach to search for clues about neuronostatin's actions. Large doses of neuronostatin were injected intraperitoneally (i.p.) in mice or via an intracerebroventricular (i.c.v.) cannula in rats and tissues screened for the induction of c-Fos and c-Jun expression. Early gene expression was detected in CNS sites, pancreatic alpha cells, chief cells of the gastric mucosa and in intestinal villi. These results allowed us to extend our studies to in vitro and in vivo bioassays used to characterize neuronostatin's biologic actions.

Neuronostatin altered growth cone migration of cultured cerebellar granule cells, activated early gene expression in KATOIII cells a (a gastric tumor cell line we would subsequently use to identify the neuronostatin receptor), and glucagon release from isolated rat and mouse pancreatic islets [5,7]. In isolated cardiac myocytes and Langendorf whole heart preparations neuronostatin exerted negative chronotropic and inotropic effects [2,9]. Importantly, the myocyte mechanical effects were prevented by pretreatment with the protein kinase A inhibitor (H-89) and the Jun-N-terminal kinase (JNK) inhibitor SP6000125 [2]. These were the first signaling data that suggested the neuronostatin receptor was G protein-coupled. Direct membrane effects of neuronostatin were observed in hypothalamic slice cultures [7]. Thus multiple pharmacologic effects of the peptide were observed in a variety of cells and tissues, mirroring its wide expression patterns. But could significant actions be demonstrated in vivo?

Our studies have focused upon cardiac, CNS and pancreatic sites of action in vivo. Our initial cellular and organ systems based observations of negative chronotropic and inotropic effects of neuronostatin have been verified in whole animal studies in which a bolus injection of the peptide into adult, male C57 BL/6 mice suppressed cardiac contractile function as monitored by echocardiography [15]. Mechanistic changes associated with the actions of neuronostatin include decreased phosphorylation of sarcoplasmic reticulum calcium ATPase (SERCA) and phospholamban (PLB) and activation of AMP-dependent protein kinase (AMPK).

Was it possible that the myocyte effects of neuronostatin were expressed via interaction with a somatostatin receptor? Somatostatin exerted similar mechanistic actions on cultured myocytes; however, the protein kinase C inhibitor, chelerythrine, which inhibited somatostatin's action, failed to alter the response to neuronostatin [2]. In addition, unlike neuronostatin's effects, somatostatin's action on myocyte contractility was not prevented by pretreatment with PKA or JNK inhibitors [2]. These results, when considered along side our previous demonstration that neuronostatin did not displace labeled somatostatin from any of the five known somatostatin receptors [7] led us to hypothesize that neuronostatin's biologic actions were unique from those of its co-expressed partner, somatostatin.

When administered into the central nervous system, neuronostatin exerted pharmacologic actions unique from those of somatostatin. Central (i.c.v.) administration of neuronostatin increased mean arterial pressure (MAP), without altering spontaneous locomotor activity, and suppressed light-entrained feeding and water drinking in adult male rats [7,12]. The anorexigenic action of neuronostatin was prevented by pretreatment of the animals with the melanocortin antagonist SHU9119, indicating recruitment of pro-opiomelanocortin (POMC) producing neurons [14]. SHU9119 pretreatment also blocked the central hypertensive action of neuronostatin [12]. We now believe that the anorexigenic