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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 minireview 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.

Keywords

Neuropeptides; Hypothalamus; Pituitary; Receptors

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

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The Senior Author was fortunate to publish an original research article in the inaugural, Spring 1980, issue of *Peptides* [6] reporting that luteinizing hormone releasing hormone extracted from the organum vasculosum lamina terminalis was similar chromatographically to that harvested from the median eminence. While this was not an earth-shattering discovery, it was important for our understanding of the hypothalamic circuitry controlling reproduction. The Editor, Dr. Abba Kastin, recognized the potential importance of the finding, even given the paper's brevity and its limited audience, and he decided to give us a voice. We have been loyal to Dr. Kastin ever since. The most important contribution of Dr. Kastin to our work has been his honesty and his encouragement.

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 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 bioinformatics-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 used to co-localization of neuronostatin and somatostatin and develop 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 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 actions of neuronostatin are secondary to the central hypertensive activity.

The central hypertensive action of neuronostatin was further characterized to be biphasic in nature, with rapid and transient elevation of sympathetic nerve activity, followed by elevation in the release of vasopressin from the neurohypophysis [12]. This pharmacologic action of neuronostatin became important to our subsequent studies on physiologic relevance [13].

In the pancreas, neuronostatin is produced along with somatostatin in the delta cells and like somatostatin can inhibit glucose-stimulated insulin secretion from isolated pancreatic islets [5, 7]. However, the effect of neuronostatin on beta cell response to glucose is indirect, requiring the presence of the alpha cell. In both whole islets and transformed alpha cell lines, neuronostatin increases glucagon mRNA levels and release [5]. The in vitro actions of neuronostatin in isolated islets have a correlate in the whole animal. Pretreatment with neuronostatin prior to a glucose challenge in adult, male rats resulted in delayed glucose clearance from plasma, most likely related to the observed reduction in insulin secretion compared to controls [5]. The alpha cell actions of the peptide also are consistent with G protein coupled receptor signaling, because we neuronostatin increased phosphorylation of the catalytic subunit of PKA (Elrick et al. manuscript under review).

Are the pharmacologic effects of neuronostatin physiologically relevant?

While we had made significant progress in our efforts to understand the cardiac, CNS and pancreatic actions of neuronostatin, whether these pharmacologic effects were physiologically relevant remained unknown. Selective antagonists were not yet available for the peptide and thus we decided to attempt a high risk/high reward approach to this question. We reasoned that several of the peptide's effects were secondary to recruitment of signaling cascades associated traditionally with G protein coupled receptor activation. Thus we hypothesized that the cognate neuronostatin receptor was one of the more than 100 orphan G

protein coupled receptors included in the IUPHAR database [8]. Using a novel strategy developed by Dr. Yosten, cell lines responsive to neuronostatin were screened for the expression of all orphan GPCRs. This “Deductive Reasoning Strategy” was created for the purpose of deorphanizing the neuronostatin receptor [13], and has been employed subsequently [10] to identify a receptor for connecting peptide (C-peptide). Four cell types previously demonstrated to be responsive to neuronostatin were examined for expression of the panel of orphan GPCRs. All four cell types shared in common the expression of four of these orphans, GPR56, GPR107, GPR146, and GPR160 [13]. This strategy resulted in the identification of a manageable number of orphan receptors to screen using siRNA depletion approaches. We examined the effects of siRNA-induced depletion of the candidate receptors in KATOIII cells on c-Fos expression following neuronostatin treatment. Compromise of GPR56 expression failed to alter the c-Fos response to neuronostatin, thus eliminating GPR56 from our list of candidates. This in the end was a fortuitous control, because during our work, GPR56 was identified to be the unique, collagen III receptor [4]. Similarly, reduction of the expression of GPR146 failed to alter KATOIII cell responses to neuronostatin, eliminating that GPCR from our list. However, when we compromised mRNA levels for GPR107, the ability of neuronostatin to increase cFos mRNA in KATOIII cells was significantly and virtually completely abrogated [13]. Thus GPR107 became our prime candidate for the cognate receptor for neuronostatin. This was further validated by recent studies, still in progress in which we have identified the requirement of GPR107 expression for the action of neuronostatin to increase proglucagon mRNA levels in both the transformed alpha cell line and whole, isolated pancreatic islets (Elrick et al. 2015, Manuscript in Review).

Can the requirement for activation of GPR107 by neuronostatin be demonstrated in vivo? We returned to our in vivo model of neuronostatin's CNS hypertensive action to answer this question. Considering that i.c.v. administration of the peptide increased MAP, we reasoned that siRNA induced compromise of GPR107 mRNA levels would prevent the action of exogenously administered peptide. Indeed, i.c.v. administration of GPR107 siRNA decreased mRNA levels for the receptor in rat hypothalamus and this was associated with a failure of those animals to respond to a dose of neuronostatin that in control (eGFP) siRNA-administered animals significantly elevated MAP [13]. Reviewers of that manuscript suggested that this was a non-specific effect, stimulating us to repeat the protocol and examine if another non-neuronostatin-related peptide, angiotensin II, would still elevate MAP in GPR107 siRNA-treated rats, and indeed it did. Thus the failure of neuronostatin to elevate MAP in the GPR107 siRNA pretreated rats was not due to some non-specific effect.

This verified that the pharmacologic action of neuronostatin to act within the CNS to increase MAP required the presence of GPR107, but it still did not address the issue of physiologic relevance. We conducted one more protocol in GPR107 siRNA pretreated rats and demonstrated compromise of baroreflex function in those animals, thus supporting two hypotheses: 1) that endogenously produced neuronostatin is an important messenger in the neural circuitry communicating baroreflex activation in the CNS, and 2) that the action of neuronostatin requires the expression of GPR107 [13].

What remains to be established in terms of the association of neuronostatin with GPR107? We recently have demonstrated by dual and triple labeling immunohistochemistry that neuronostatin does co-localize with GPR107 on its target tissues (Figure 1). These findings provide further support that GPR107 is the neuronostatin receptor; however, we have not expressed GPR107 in a null cell line for the purpose of determining receptor kinetics, nor have we characterized the receptor kinetics in cells that endogenously express GPR107. We leave those projects for investigators interested in pharmacokinetics and mimetic development and welcome their interest and efforts. Instead we seek now to further understand the physiological consequences of the tissue specific loss of neuronostatin action in selective tissues, for example the endocrine pancreas, and the potential for neuronostatin agonists and antagonists to be developed for use as a therapeutic in variety of disorders of glucose homeostasis or cardiovascular function.

The identification and characterization of the physiological relevance of neuronostatin serves as a template, a roadmap actually, for the study of novel targets for therapeutic intervention. We believe that this is just the start for the identification of new peptide hormones, as there remain numerous candidates on Aaron Hsueh's list of previously unidentified peptide sequences encoded in the mammalian genome. One of those, phoenixin, recently was demonstrated to be a novel regulator of reproductive function [11]. Using the methodical approaches and strategies described above we are working now to identify its sites of production, full spectrum of biologic effects, mechanisms of action, cognate receptor and role in the control of mammalian reproduction. The journal *Peptides* and its longstanding and authoritative Editor, Dr. Abba Kastin, have had a major, positive impact on the growth of our knowledge of peptide biology and their legacy will endure into the future as we continue to discover and characterize previously unrecognized and potentially important peptides and their receptors.

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Highlights

- Bioinformatic analyses revealed the novel hormone, neuronostatin
- Neuronostatin is encoded in the preprosomatostatin prohormone
- Neuronostatin has biologic actions unique from those of somatostatin
- GPR107 was determined to mediate the effects of neuronostatin
- Pancreas is a promising target for therapeutic use of neuronostatin

Figure 1. Colocalization of neuronostatin and GPR107 in human pancreas tissue

Human pancreas section obtained during routine autopsy at Saint Louis University Hospital were exposed to exogenous neuronostatin (30 nM), then fixed and stained using antibodies directed against GPR107 (green), neuronostatin (red), and glucagon (blue). A single islet is shown in which multiple instances of triple co-localization (white) are observed in the merged image (lower left panel), indicating that neuronostatin and GPR107 co-localize on alpha cell membranes.

Non-transgenic approaches to the understanding of a peptide's physiological relevance: Compromise of Function and Production

Table 1

Compromise of Function	Target	References
Passive Immunoneutralization	Corticotropin releasing factor	Ono et al. 1985
	Oxytocin	Samson et al. 1986
	Neuropeptide B	Samson et al. 2004
Small interfering RNAs (siRNAs)	Obestatin	Samson et al. 2008
	Neurostatin receptor (GPR107)	Yosten et al. 2012
	C-peptide receptor (GPR146)	Yosten et al. 2013
Cytotoxin-based cell targeting	Oxytocin receptors	Samson et al. 1992
		Blackburn et al. 1993, 1995
	Natriuretic Peptide receptors	Samson et al. 1985

Compromise of Production	Target	References
Antisense oligonucleotides	Adrenomedullin	Samson et al. 1999
	Nesfatin-1	Yosten et al. 2012
	Adrenomedullin	Taylor and Samson 2002, 2003
Small interfering RNAs (siRNAs)		White and Samson 2009
	Neuropeptide W	Pate et al. 2013
	Phenixin	Yosten et al. 2013