On the origin of growth-associated protein-43 (GAP-43) immunoreactive processes present in the rat anterior pituitary

by Jason Michael Kuhl

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

Montana State University

Abstract:

Recent studies have demonstrated the presence of an axon plexus immunoreactive (ir) for the neuronal growth-associated phosphoprotein-43 (GAP-43), a membrane-bound phosphoprotein implicated in axonal growth, within the rat anterior pituitary (AP). Though these fibers are implicated in modulating the hypothalamo-pituitary-adrenal (HPA) axis, their source of origin remains unknown. The current studies used anterograde and retrograde neural tracing, surgical ablation, and immunocytochemistry in an effort to elucidate the source(s) of GAP-43-ir processes in the adult male rat AP. In the first experiment the lipophilic tracer dialkylcarbocyanine (DiI) was used to determine if the GAP-43-ir processes were originating from the trigeminal ganglia, and therefore of sensory origin. Due to diffusion of the DiI and very little labeling of structures in the AP this study was determined to be inconclusive: The second experiment looked at possible autonomic innervation, and involved the bilateral removal of the superior cervical ganglia (SCGX). Immunocytochemical staining for GAP-43 after SCGX revealed no decrease in the density of immunoreactive structures, strongly suggesting that the GAP-43-ir structures are not sympathetic in nature. The last two experiments involved retrograde tracing of the AP using either DiI or Fluorogold (FG). DiI tracing, again, appeared to be inconclusive due to dye diffusion. However, retrograde tracing using FG was accomplished, and revealed specific labeling of neuronal cell bodies within the medial habenular nuclei (MHb). This staining does not appear to be artifactual. If existence of this novel central projection is confirmed, it would provide new insights into the mechanisms by which the MHb modulates the stress response.

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Jason Michael Kuhl

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Recent studies have demonstrated the presence of an axon plexus immunoreactive (ir) for the neuronal growth-associated phosphoprotein-43 (GAP-43), a membrane-bound phosphoprotein implicated in axonal growth, within the rat anterior pituitary (AP). Though these fibers are implicated in modulating the hypothalamo-pituitary-adrenal (HPA) axis, their source of origin remains unknown. The current studies used anterograde and retrograde neural tracing, surgical ablation, and immunocytochemistry in an effort to elucidate the source(s) of GAP-43-ir processes in the adult male rat AP. In the first experiment the lipophilic tracer dialkylcarbocyanine (DiI) was used to determine if the GAP-43-ir processes were originating from the trigeminal ganglia, and therefore of sensory origin. Due to diffusion of the DiI and very little labeling of structures in the AP this study was determined to be inconclusive. The second experiment looked at possible autonomic innervation, and involved the bilateral removal of the superior cervical ganglia (SCGX). Immunocytochemical staining for GAP-43 after SCGX revealed no decrease in the density of immunoreactive structures, strongly suggesting that the GAP-43-ir structures are not sympathetic in nature. The last two experiments involved retrograde tracing of the AP using either DiI or Fluorogold (FG). DiI tracing, again, appeared to be inconclusive due to dye diffusion. However, retrograde tracing using FG was accomplished, and revealed specific labeling of neuronal cell bodies within the medial habenular nuclei (MHb). This staining does not appear to be artifactual. If existence of this novel central projection is confirmed, it would provide new insights into the mechanisms by which the MHb modulates the stress response.

INTRODUCTION

Previous studies done by Paden et al (1994) have described the presence of an extensive plexiform network of fine beaded nerve fibers within the parenchyma of the anterior lobe (AP) of the rat pituitary. These axons were visualized through peroxidase immunocytochemical localization of the neural-specific growth-associated protein-43 (GAP-43, also known as B-50, F1, pp46, p57, and neuromodulin), a membrane-bound phosphoprotein found in axonal growth cones, and implicated in axonogenesis and synaptic remodeling (Benowitz and Perrone-Bizzozero, 1991; Gispen et al., 1991; Meiri et al., 1986; Skene, 1989). The presence of GAP-43 suggested that the axon terminals in the AP are capable of morphological plasticity. Supporting this hypothesis, Lu et al (1995) reported that the density of GAP-43 immunoreactive (GAP-43-ir) axons in the rat adenohypophysis was significantly increased 4 days after bilateral removal of the adrenal glands (ADX). Subsequently, this laboratory demonstrated that the increase in GAP-43-ir was indicative of axonal collateral sprouting as fibers grew to specifically contact over 90% of corticotrophs two weeks after ADX, when these cells are known to be mitotically active and hypertrophic (Paden et al., 1998). The extent, celerity, and selectivity of axonal sprouting in response to ADX strongly suggests that GAP-43-ir axons are of functional significance in the AP, and likely involved in the regulation of the stress response produced by the activation of the hypothalamo-pituitary-adrenal (HPA) axis. However, because GAP-43 is found in a wide variety of nerve fibers in both the central

nervous system (CNS) and peripheral nervous system (PNS) (Bendotti et al., 1991; Benowitz et al., 1988; Del Fiacco et al., 1994; Kruger et al., 1993; Sharkey et al., 1990; Stewart et al., 1992; Yao et al., 1993), its presence within axons innervating the AP reveals little about their possible origin.

The specific goal of this project was to determine the origin of GAP-43-ir axons found in the parenchyma of the rat AP through the use of four different experimental designs. The first study involved post-mortem anterograde tract tracing of the trigeminal nerve (CN V) using the fluorescent dialkylcarbocyanine lipophilic tracer DiI. This experiment was undertaken to test the hypothesis that the GAP-43-ir fibers were sensory in origin. The second experiment involved the bilateral surgical removal of the superior cervical ganglia (SCGX) in an effort to determine if the GAP-43-ir fibers were of sympathetic origin. The third experimental design utilized DiI for post-mortem retrograde tracing of the axons terminating in the rat AP to determine if the GAP-43-ir fibers originated in the brain. The fourth experiment involved retrograde axonal tracing after iontophoresis of Fluorogold (FG) into the ventral AP of live rats, in order to investigate all three previously described potential sources of fibers.

Upon completion of the fourth experiment FG was found to be bilaterally localized within the medial habenular nuclei (MHb). This labeling does not appear to be artifactual, with FG specifically localized within neuronal cell bodies. Furthermore, new evidence suggesting that the MHb is involved in the stress response (Andres et al., 1999; Scheibel, 1997), suggests a possible link with previous studies from this laboratory showing that increased density of GAP-43-ir in the AP is associated with activation of the HPA axis and increased corticotropic activity (Paden et al., 1998).

LITERATURE REVIEW

Development of the Pituitary

The hypophysis (pituitary gland) forms during development from two separate primordial ectodermal outgrowths just beneath the brain at the level of the mesodiencephalic junction. Rathke's pocket is one of these outgrowths, arising from the stomodeal ectoderm of the buccal cavity. The other primordial outgrowth is the infundibular process, arising from the neural ectoderm in the floor of the diencephalon. Rathke's pocket grows along the midline toward the diencephalic floor to secondarily unite with the infundibular process at the level of the diencephalon.

In time the infundibular process develops into the constitutive pars neuralis (neurohypophysial subunit, or neural lobe) of the adult pituitary. Concomitantly, Rathke's pocket grows into a double-layered cup, partially encapsulating the infundibular process of the future pars neuralis. Later in development, the anterior portion of this cup thickens to form the sinusoidal tissue and vasculature known collectively as the pars distalis (adenohypophysial subunit, or anterior lobe) giving rise to the glandular-like appearance of the pituitary. The thin layer of Rathke's pocket adjacent to the pars neuralis ends up forming the last of the three lobes present in the adult organism and is respectively named the pars intermedia (intermediate lobe).

The neural stalk that joins the pituitary gland to the brain is formed mainly by the axons of the vasopressin (AVP) and oxytocin (OX) magnocellular secretory neurons originating in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus. The vast majority of AVP and OX axons come to terminate in the neural lobe. In fact, this innervation is so prevalent that the morphology of the developing neural lobe is almost completely characterized by the arborization of these magnocellular axons, along with a relatively small number of glia (pituicytes and microglia) and a rich bed of fenestrated capillaries. In contrast, the histology of the developing anterior lobe consists of six secretory cell types that are capable of producing at least eight separate hormones, supporting glial-like folliculostellate cells, and a dense capillary plexus.

The Functional Pituitary and the Hypothalamic-Pituitary-Adrenal Axis

The neural lobe, intermediate lobe, and anterior lobe have unique and very distinct functions in both the developing and adult mammalian organism. The neural lobe forms a neurosecretory tissue releasing the polypeptides AVP (also known as antidiuretic hormone) and OX. The intermediate lobe secretes melanocyte-stimulating hormone and beta-endorphin. The anterior lobe is composed of a variety of secretory cell types which include: somatotrophs (growth hormone), lactotrophs (prolactin), corticotrophs (adrenocorticotropic hormone), gonadotrophs (lutenizing hormone and follicle stimulating hormone), and thyrotrophs (thyroid stimulating hormone) (Bennett and Whitehead 1983; Nakane, 1970). All eight of the hormones produced by the secretory cells found in the anterior lobe act as tropic effectors for other endocrine

glands, and are involved in the positive and negative feedback loops that assist in the modulation and/or maintenance of an organism's internal environment.

Of particular relevance to this study are the negative feedback loops involved in regulation of the HPA axis (Figure 1). This feedback system regulates the levels of glucocorticoids (corticosterone in rodents, cortisol in most other mammals) found in mammals. Increased activity of the HPA axis is initiated by the hypothalamic release of corticotropin-releasing factor (CRF) in response to physiological and/or environmental stress. The secreted CRF enters the AP via the portal vasculature of the pars tuberalis, thereby functioning as an activator for the secretion of adrenocorticotrophin (ACTH) by the AP. The release of ACTH into the circulatory system, in turn, stimulates the production and secretion of gluococorticoids by the adrenal gland. The feedback loop is completed by the increased systemic levels of glucocorticoids stopping the hypothalamic release of CRF, leading to the negative feedback inhibition of ACTH release. Glucocorticoid negative feedback is also exerted directly at the level of the corticotrophs.

The HPA axis and its control over glucocorticoid levels is of notable physiological importance to the mammalian organism. The corticosteriods secreted during stress exert powerful anti-insulin effects on protein and carbohydrate metabolism, induce synthesis of glucose by the liver, activate enzymes in muscle that are involved in the catabolism of proteins, stimulate lipolysis in adipose tissues, and suppress the immune system (Porth, 1990).

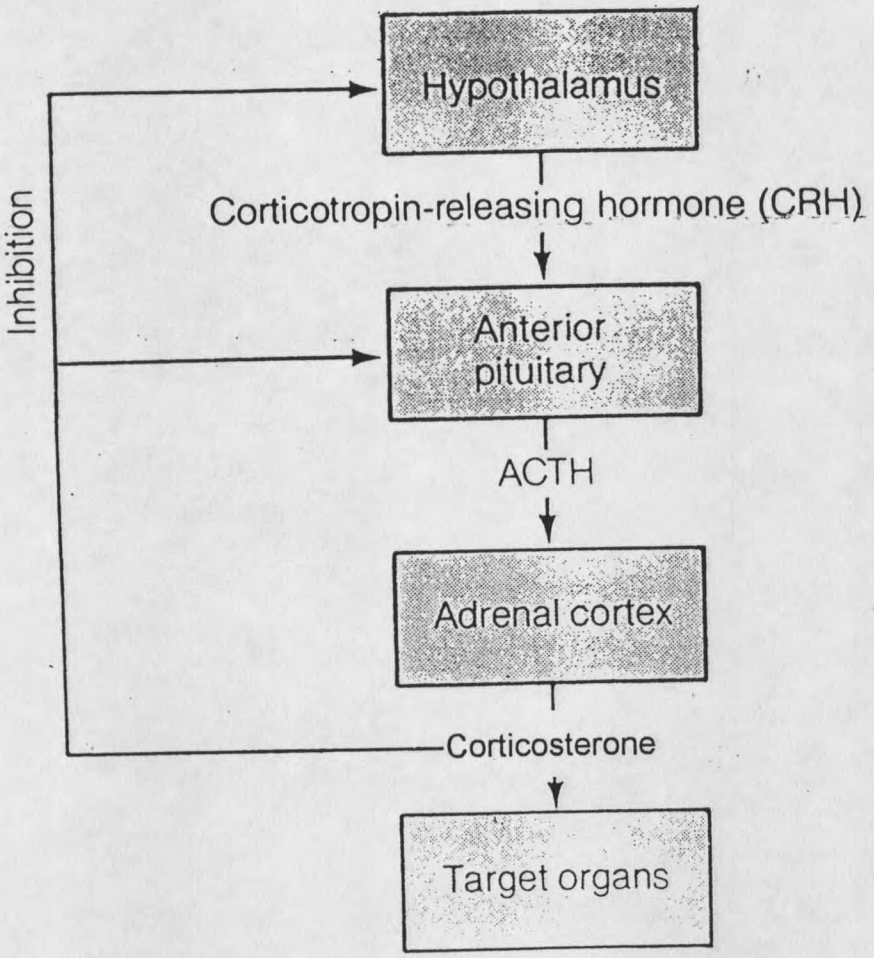

Figure 1. The hypothalamic-pituitary-adrenal (HPA) feedback system regulating circulating glucocorticoid (corticosterone) levels. Increased corticosterone levels incite a negative feedback inhibition of ACTH release by the AP.

During periods of chronic stress the HPA axis of rats is altered, resulting in tonic elevations of plasma corticosterone of varying magnitude (Dallman 1993). Chronic stress is also accompanied by a slightly elevated level of plasma ACTH, increased hypothalamic CRF, and decreased pituitary CRH receptors (Tizabi and Aguilera 1992). Interestingly, the increased level of plasma corticosterone does not inhibit the HPA axis, which is at least fully responsive, if not hypersensitive, to acute or novel stressors (Dallman 1993; Tizabi and Aguilera 1992). The maintenance of HPA axis sensitivity during sustained chronic stress is currently believed to be due to stress induced changes in the input to CRH neurons and in the neurons themselves.

Innervation of the Anterior Pituitary

Throughout most of the 20th century it had been thought that the anterior pituitary was only sparsely innervated by autonomic nerve fibers, and by a few additional fibers of unknown origin (Aleshin, 1964; Dandy, 1913; Friedgood, 1970; Green, 1966). The sparse innervation of the adenohypophysis by the autonomic nervous system is believed to consist mainly of postganglionic ascending fibers from the superior cervical ganglia (SCG). These fibers have been reported to directly innervate the vascular and parenchymatous elements of the AP by way of the cavernous plexus (Friedgood, 1970). It has also been shown that in canines and felines, sympathetic nerve fibers extend off the carotid plexus to innervate the adenohypophysial subunit (Dandy, 1913).

More recently, since the advent of immunocytochemistry, the innervation of the mammalian anterior pituitary gland has been reinvestigated. Convergent work from several groups demonstrated a fairly substantial innervation of the AP by peptidergic nerve fibers in several mammalian species. Currently the rat AP is believed to be innervated by somatostatin fibers (Westlund et al., 1983), serotonin fibers (Westlund and Childs, 1982), and pituitary adenylate cyclase activating peptide (PACAP) fibers (Mikkelsen et al., 1995), while fibers immunoreactive for both substance P (SP) and calcitonin gene-related peptide (CGRP) have been found present in the AP of rats, dogs, macaques, and humans (Gon et al., 1990; Ju et al., 1991; Ju et al., 1993; Ju and Liu, 1989a; Ju and Liu, 1990; Ju and Zhang, 1990; Ju and Zhang, 1992; Liu and Ju, 1988; Lu et al., 1995; Mikkelsen et al., 1989; Skofitsch and Jacobowitz, 1985; Tschopp et al., 1985). Another set of processes immunoreactive for GAP-43 have also been localized to the AP of the rat (Lu et al., 1995; Paden et al., 1994; Paden et al., 1998). However, their exact phenotype has yet to be elucidated and doing so is one of the goals of this project. Many of these fibers have been found to be in close contact with a variety of AP gland cells including lactotrophs, somatotrophs, corticotrophs, gonadotrophs, and thyrotrophs (Gon et al., 1990; Ju et al., 1991; Ju and Liu, 1989b; Liu et al., 1996; Mikkelsen et al., 1989; Paden et al., 1994; Paden et al., 1998; Westlund et al., 1983). Furthermore, electron microscopy has been used to show that some of these fibers form synaptic contacts with the AP secretory cells (Ju and Zhang, 1990; Ju and Zhang, 1992; Liu et al., 1996).

GAP-43 as a Marker of Innervation and Collateral Axonal Sprouting

GAP-43 (also known as B-50, F1, pp46, p57, and neuromodulin) is a 24 kD membrane-associated phosphoprotein present in the axonal growth cones of a wide variety of nerve fibers found throughout both the CNS and PNS (Benowitz et al., 1988; Del Fiacco et al., 1994; Sharkey et al., 1990; Stewart et al., 1992). It is expressed at high levels during the development of the nervous system and is increased during periods of axonal regeneration and morphological plasticity in adults (Benowitz and Perrone-Bizzozero, 1991; Gispen et al., 1991; Meiri et al., 1986; Skene, 1989). The temporal correlation existing between axonal outgrowth and increased GAP-43 expression suggests that GAP-43 plays a functional role in axonogenesis, maintenance of the functional growth cone, and synaptic remodeling (Benowitz and Perrone-Bizzozero, 1991; Benowitz et al., 1981; Gispen et al., 1991; Meiri et al., 1986; Skene, 1989; Skene and Willard, 1981a; Skene and Willard, 1981b; Skene and Willard, 1981c).

Special interest in the function of GAP-43, as well as other growth-associated proteins, has arisen in response to the observation that regeneration and/or sprouting is often accompanied by a specific 20- to 100-fold increase in synthesis of these proteins, and their subsequent axoplasmic transport into the growing axon (Kalil and Skene, 1986; Skene and Willard, 1981a). The presence of GAP-43 within certain areas of the damaged CNS is of particular significance because axoplasmic transport is not normally found to be restored in the injured axons--a likely factor in the inability of most CNS neurons to regenerate and/or undergo morphological changes. Therefore, the re-institution of axoplasmic transport of GAP-43, and its presence in the axonal growth cone in response

to nerve insult in both the CNS and PNS makes GAP-43 an ideal marker for studying the sprouting and regenerative capacity of axons in the nervous system.

Little is known about the true functional significance of GAP-43 within the mammalian nervous system above and beyond its localization to the membrane fraction of the growth cone (it was not found to be present in the cytoskeleton) (Meiri and Gordon-Weeks, 1990). GAP-43 has been shown to behave like an integral membrane protein in detergents and salts (Skene and Willard, 1981a; Dosemeci and Rodnight, 1987). However, the primary sequence of GAP-43 contains no membrane-spanning domains (Basi et al., 1987, Karns et al., 1987). It has been observed that GAP-43 is highly phosphorylated within the neuron when the cell is undergoing depolarization and exocytosis (Dekker et al., 1990; Van Hooff et al., 1989). Furthermore, the kinase C phosphorylation of GAP-43 appears spatially restricted to the growth cone after the protein has undergone axoplasmic transport in its dephosphorylated state (Meiri et al., 1991). Thus there is believed to exist a temporal delay between the onset of GAP-43 expression concurrent with initiation of axonal outgrowth, and the subsequent phosphorylation of GAP-43 believed to be involved in stabilizing the growth cone. Currently it is thought that the induction of GAP-43 is a critical step involved in the conversion of a neuron to a competent metabolic state in which it is capable of axonal elongation (Skene and Willard, 1981b; Skene and Willard, 1981a). If this is indeed occurring, failure of neurons to elevate their levels of GAP-43 protein in response to insult or injury would be a limiting factor in the neuron's ability to regenerate and/or sprout an axon.

GAP-43 has been over-expressed in the neurons of adult transgenic mice (Aigner and Caroni, 1995). Axonal sprouting and terminal arborization were seen to be greatly potentiated compared to normal mice in response to paralyzing the neuromuscular junction, crushing the sciatic nerve, or lesioning the dorsal root ganglia (DRG). However, these transgenic mice also exhibited spontaneous sprouting of axon collaterals at the neuromuscular junction and in the terminal field of hippocampal mossy fibers (Aigner and Caroni, 1995). The hyperphysiological presence of neuronal GAP-43 in the transgenic model is believed to assist in stabilizing spontaneous lamellar and filopodial structures at the axonal terminal (Robbins and Polak, 1988), thereby causing the accumulation of new nerve sprouts in an environment normally devoid of such activity. Thus, it could be hypothesized that up-regulation of GAP-43 in response to injury in a normal organism could also act to stabilize spontaneously forming lamellar and filopodial processes in the regenerating axon.

GAP-43 in the Anterior Pituitary

Earlier studies from this laboratory have demonstrated the presence of an axonal plexus immunoreactive for GAP-43 in the rat AP (Paden et al., 1994). At the level of the light microscope the staining appears as large varicose nerve bundles, likely made up of multiple smaller axons, universally scattered throughout the lobe (Figure 2). Numerous fine beaded axon processes can be seen to course individually through the coronally cut Vibratome sections. However, the majority of the GAP-43 immunoreactivity

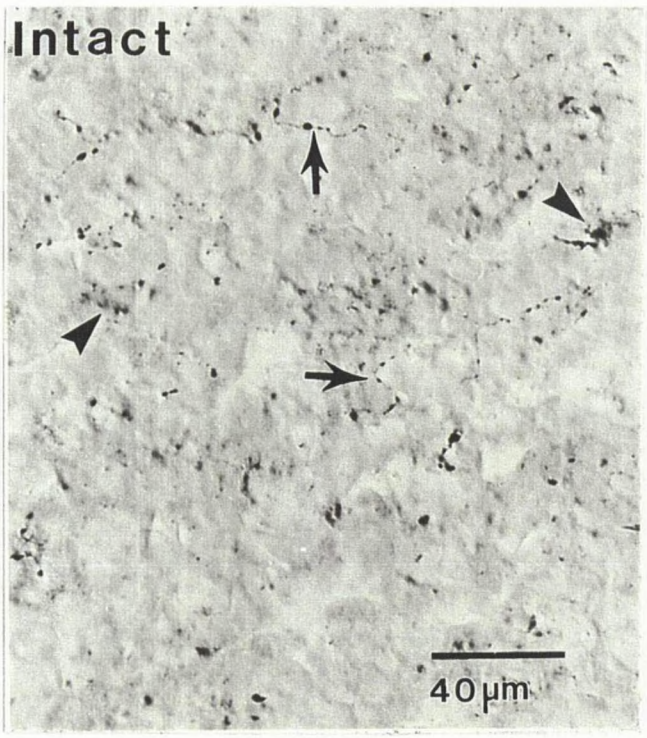

Figure 2. GAP-43 immunoreactivity in a peroxidase stained 40 µm thick section of the intact rat AP. Both fine beaded axons (arrows) and punctate clusters of putative axon terminals (arrowheads) are visible. Magnification is 450X.

(GAP-43-ir) appears as intense punctate staining apparently surrounding a population of secretory cells within the AP. As previously described, the presence of GAP-43 strongly suggests that these apparent axonal terminals are capable of morphological plasticity.

In support of this hypothesis, the density of GAP-43-ir axons in the rat AP is significantly increased 4 days after bilateral adrenalectomy (ADX) (Figure 3 and Lu et al., 1995). Subsequently this laboratory demonstrated that the increase in GAP-43-ir appeared indicative of axonal sprouting, as fibers grew to specifically contact over 90% of corticotrophs two weeks after ADX (Figure 4), a time when these cells are mitotically active and hypertrophic (Paden et al., 1998). The extent and selectivity of the apparent collateral sprouting in contacting corticotrophs strongly suggest that these fibers are of functional significance, giving rise to the hypothesis that the innervation of corticotrophs by GAP-43-ir processes may act as an additional control mechanism that is highly plastic in response to activation of the HPA axis (see below).

Since GAP-43 has previously been shown to facilitate neurotransmitter release by axons (Dekker et al., 1989; Ivins et al., 1993), it could be speculated that these processes are somehow involved in modulating the secretion of ACTH. The fact that plasticity appears as the rule rather than the exception in the HPA axis is of great importance to understanding the physiological mechanisms involved in controlling and modulating stress. The ability of the GAP-43-ir fibers found present in the AP to undergo morphological plasticity would seem to be a requisite for effective innervation of the constantly changing population of corticotrophs.