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13. ABSTRACT (Maximum 200 words)

The goals of the study were to characterize the sequence of events involved in the hypothalamic-pituitary responses to cold stress. In the hypothalamus, studies focused on Arginine vasopressin neurons and changes in expression of mRNA following cold and novel environment exposure. In the pituitary, studies focused on AVP and CRH target cells, including corticotropes, thyrotropes, and intermediate lobe cells. Changes in size, granulation, expression of mRNA, and binding sites for AVP and CRH were detected. *In vitro* studies of second messenger effects on CRH and AVP binding provided more clues about mechanisms of action. Finally, a new cell type that changed to a multihormonal corticothyrotrope under the influence of AVP was described. It could provide an efficient means of stimulating both the thyroid and the adrenal. Studies implicating locally produced epidermal growth factor (EGF) as another modulatory agent in the stress response were also done because EGF stimulates corticotropes. Cells producing EGF increased expression of EGF mRNA after exposure to cold. In summary, these studies identified four critical hormones that interact with each other in the control of pituitary responses to cold. These included: AVP, CRH, thyrotropin releasing hormone (TRH) and epidermal growth factor.

14. SUBJECT TERMS

corticotropes, thyrotropes, cold stress, anterior pituitary, paraventricular nucleus, arginine vasopressin, adrenocorticotropin, Corticotropin releasing hormone, in situ hybridization, immunocytochemistry, image analysis, RIA, rats

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FINAL REPORT ON CONTRACT N00014-88-K-0016

PRINCIPAL INVESTIGATOR:Gwen V. Childs, Ph.D.

CONTRACTOR:THE UNIVERSITY OF TEXAS MEDICAL BRANCH

START DATE:1 October 1987-01 year; 1 October 1988-02 year; 1 October 1989--03 year; Extension 1 October, 1990-September 30, 1991.

RESEARCH OBJECTIVE: The overall research objective was to learn more about the control and mediation of responses to cold stress by pituitary anterior lobe cells. Studies were done to determine if there were changes in the anterior lobe corticotropes and thyrotropes including percentages, cell areas, expression of mRNA (for proopiomelanocortin) and antigens. Expression of mRNA and antigen activity was measured by image analysis. To learn more about the control of corticotropes during stress responses, changes in the percentages of target cells for corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) were also investigated. These studies were followed by more specific studies of the effect of second messengers on CRH binding.



Studies of the function of AVP in modulation of corticotrope and thyrotrope responses included identification of AVP binding sites in the pituitary by dual labeling for AVP and adrenocorticotropin and thyrotropin. The effect of other secretagogues on AVP binding was also studied. Finally, the effect of exposure to cold or novel environment stress on the expression of AVP mRNA and antigens by different cells in the hypothalamus was investigated.

During the extension period, the studies were focused on the role of a newly discovered secretagogue for corticotropes, epidermal growth factor (EGF). This polypeptide has recently been found in pituitary cells and therefore, it may be a local stimulatory agent. After its stimulatory role on hormone synthesis and secretion in corticotropes was confirmed, the effect of cold and novel environment stress on EGF-containing pituitary cells was investigated.

PROGRESS: Phase 1: Development of stress tests. The first group of studies (during the 01 year) were focused on establishing the baseline parameters for the experimental design. Tests of acclimation and handling conditions were done extensively to prevent non-specific stresses. The results showed that housing the rats 4-5 per cage X 3 cages helped prevent the stress associated with isolation or with rats housed in small groups, like pairs. During acclimation, the investigators identified the dominant rat in the cage and eliminated him from the study because of his predictable higher ACTH levels. After 7-10 days of handling

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before 10 AM, three investigators removed one rat from each of the cages. Rat A was taken to a separate room and quickly (within seconds) sacrificed by guillotine. This protocol prevented rapid elevation of ACTH and kept baseline levels under 150 pg/ml. Rat B was taken to a separate room and placed in a cage in the refrigerator (4 C) (cold stress). Rat C was placed in a box (novel environment exposure).

Rats B and C were killed by guillotine after 15-30 min of the cold or novel environment exposure. Trunk blood from all three rats was collected for adrenocorticotropin (ACTH) radioimmunoassay. The pituitaries were removed rapidly and either fixed in Bouins fixative (for detection of antigens) or 2% glutaraldehyde (for electron microscopy). Or pituitaries were placed in growth media for dissociation and plating. The brains were either fixed in Bouins (for detection of antigens), or rapidly frozen in liquid nitrogen for detection of AVP mRNA. The experiments were repeated so that a minimum of 5 rats/group were sampled. Repeats also were done to provide enough material for the various types of tests on tissues and cells.

Phase 2. Effect of stress on anterior lobe cells, *in vitro* studies. The neurointermediate lobe was separated from the anterior lobe. The anterior lobe cells were then dispersed, plated on glass coverslips and placed in monolayer culture. Tests showed that sites of binding to CRH or AVP were detected optimally after 12-15 h in culture. Therefore, after overnight culture, the cells were exposed to biotinylated analogs of CRH or AVP and labeled with avidin-biotin-peroxidase complex. Counts of labeled cells showed that cold stress stimulated a significant increase in the percentage of cells labeled for biotinylated CRH or AVP (40-46%). Parallel cultures were immunolabeled for 16K fragment of the pro-opiomelanocortin molecule, β -endorphin, ACTH, or thyroid stimulating hormone (TSH). Cold stress produced a 30-40% increase in the percentage of cells labeled for 16k fragment, β -endorphin, or ACTH. There was a 2--fold increase in the percentage of cells labeled for TSH (1).

Exposure to a novel environment for 30 minutes resulted in a 10-20% increase in the percentage of target cells for AVP or CRH and a 20% increase in percentages of cells labeled for 16k fragment. No increases in the percentages of cells labeled for TSH were seen after novel environment exposure (1). Therefore, even after 12-15 h in culture the pituitary cells retained the memory of the fact that the rat had been exposed to cold, or a novel environment for 30 minutes before death. The potential for expression of AVP or CRH binding, or storage of ACTH or TSH was greater in cell populations from the stressed rats. These changes correlated well with the increased blood levels assayed (2-4 fold increase after cold stress) in the serum of the stressed rats (1).

Correlative studies of the effect of stress on expression of pro-opiocortin (POMC) mRNA were conducted in subsequent years (2). The mRNA was detected with biotinylated oligonucleotide probes and streptavidin alkaline phosphatase. After 15 or 30 min of cold stress, there was a 66% or a 99% increase in the percentage of cells that expressed POMC mRNA, respectively. The shorter time caused a 117% increase in the amount of label/cell.

Interestingly, the longer time period (30 min) did not stimulate and increase in the amount of label, probably because it allowed enough time for a negative feedback by corticosterone, *in vivo*. Exposure to a novel environment caused no increases in percentages of cells expressing POMC mRNA, however, a 15 min exposure caused a 73% increase in the amount of label/cell. Thus, again even though time elapsed between stress and fixation of the cells (2-3 h), the cells retained the memory of having been stimulated *in vivo*.

Phase 3. Effect of stress on anterior lobe cells studied in tissue sections. However, to learn if these changes were evident immediately after stress, parallel studies were conducted on blocks of pituitaries prepared for electron microscopy (1). In these studies, light microscopic sections labeled for ACTH or TSH were used to determine if there were changes in cell area. The Bioquant MEG System IV was used to draw the labeled cell profiles and calculate areas of corticotropes or thyrotropes. Only cold stress caused a significant (21-22%) increase in the area of the cells which correlates with the change in serum levels of ACTH. (1) Increases in the percentages of both immunolabeled corticotropes and thyrotropes fit with the previous, *in vitro* studies.

The changes in the ultrastructure of corticotropes labeled for ACTH with gold labels were striking. After cold stress, the number of rows of secretion granules increased from one to three-four. Processes near blood vessels became filled with granules and they were longer and wider. Some of these same changes were seen in corticotropes from rats exposed to a novel environment, however the degree of change was not as striking (1).

In a recent review of the latest studies of corticotropes (3), some of the montages made of the corticotropes from the three groups of animals were drawn in a composite figure to illustrate the changes seen after stress. It is evident that even after a brief exposure to a novel environment, corticotrope lose their characteristic identifying features and become filled with secretion granules. This may be why early studies confused the identity of this cell type. More extreme stresses, like cold, cause an even greater change in morphology.

The studies of changes in POMC mRNA were also extended to include its localization on frozen sections (2). Unfortunately, the small size of the corticotropes and the small, diffuse processes precluded accurate quantification of changes in POMC mRNA in anterior lobes. Attempts to quantify the labeling as a percentage of the area of the anterior lobe showed no differences between experimental groups. This may reflect the diffuse organization of the corticotropes, or it may reflect the fact that the cells need more time to express the POMC mRNA. In the *in vitro* studies, the extra time taken in the dispersion (2-3 h) may have allowed the transcription to produce levels detectable by *in situ* hybridization. Alternatively, there could have been significant losses of POMC mRNA during the frozen sectioning. This problem was studied during the extension period.

However, the frozen sections did show dramatic increases in POMC mRNA in the intermediate lobes (which suggests that loss of mRNA was not the problem). There was a

24-fold increase in the amount of label for POMC mRNA in intermediate lobes, 30 min after cold stress. A smaller increase in the amount of label (11-fold) was seen after exposure to a novel environment. Thus, the intermediate lobe cells collectively are very responsive to these stresses. Later tests of their responses to 15 min stresses also showed significant increases in labeling for POMC mRNA.

Phase 4. Effect of cold stress on expression of mRNA and antigens by neurons containing arginine vasopressin. AVP is a secretagogue for both corticotropes and thyrotropes. This makes it a good candidate for a mediator in cold stress responses, which involve both cell types. Therefore, studies of the hypothalamic nuclei that produce AVP were initiated to learn if there were changes in expression of AVP mRNA (4). After exposure to cold for 30 min, there was a 3.5--fold increase in the label for AVP mRNA only in the paraventricular nucleus. Small but significant changes (1.2-2.3--fold) were also seen in these PVN nuclei after 30 min exposure to a novel environment. Cell bodies in the supraoptic or suprachiasmatic nuclei showed no changes.

Thus, the cold and novel environment exposures do activate a select population of AVP-producing neurons. No changes were seen in any of the neurons, or in the median eminence when the fibers were labeled for AVP antigens. It is possible that synthesis and release of AVP had accelerated so that no changes in storage could be detected. These data correlated with the studies showing increases in the percentages of AVP target cells after cold stress and implicate AVP in the mediation of the responses to certain stresses.

Phase 5. Identification of target cells for AVP in the pituitary. Although AVP stimulates the release of Thyroid stimulating hormone (TSH) or adrenocorticotropin (ACTH), it was not known if its action was mediated by direct binding to the cells. Studies prior to the initiation of this research had demonstrated ACTH secretion in a reverse hemolytic plaque assay by cells that were labeled for biotinylated AVP (5). However, further studies of binding sites were needed. Therefore, dual labels for biotinylated AVP and ACTH or TSH were developed (6).

Analyses of the AVP target cells showed that 10% of the pituitary population bound AVP; 42% contained TSH antigens and 48% contained ACTH antigens. Yet, AVP-bound only 40-60% of corticotropes or thyrotropes. Therefore, studies were begun to learn if binding was promoted or affected by pretreatment with CRH or TRH. The results showed that CRH stimulated an increase in the binding to AVP to 79% of corticotropes. An overall increase to 12.8% AVP-bound cells was seen.

CRH pretreatment of the cultures for one h did not affect the binding of AVP to thyrotropes. In contrast, TRH pretreatment stimulated binding to 75% of the TSH cells. TRH did not affect binding to corticotropes. Together, CRH and TRH stimulated an increase in the percentage of AVP-bound cells to 16% indicating that their effects were additive.

Thus, these data suggest that AVP works in concert with CRH and TRH to stimulate corticotropes and thyrotropes. More evidence for its role as a mediator in stress responses came from analysis of the binding data. When the percentages of AVP-bound cells stimulated by TRH and labeled for ACTH and TSH were added, the sum was more than 100% of AVP-bound cells. This pointed to co-storage of ACTH and TSH.

Therefore, studies that co-localized ACTH and TSH were initiated. In unstimulated populations, or in populations stimulated with CRH or TRH, only 1.5-1.7% of the pituitary cell population showed co-storage of ACTH and TSH. However, in the presence of AVP, the percentages of corticothyrotropes increased to 4.8%. This represented nearly half of the TSH cells and 40% of corticotropes. Thus, AVP mediation appears to involve activation of a multipotential cell that can respond to both the thyroid and adrenal axes. This makes it an ideal hormone for the stimulation of the pituitary during cold stress.

Phase 6. Studies of second messenger actions affecting corticotrope binding to AVP or CRH. A part of ongoing studies of binding of CRH and AVP involved tests of the activators and inhibitors of calcium channels and protein Kinase C on CRH binding. In addition tests were conducted to learn if corticosterone affected AVP or CRH binding. In the first set of studies (7), we showed that AVP potentiated CRH binding by increasing the percentage of cells labeled for biotinylated CRH. To learn if this could be mediated by second messengers, we also stimulated the cells with a phorbol ester (to stimulate protein Kinase C) and Bay K 8644 (to stimulate L-type voltage dependent calcium channels). In both cases, the percentage of CRH target cells increased. In addition, the amount of label for biotinylated CRH/cell increased (detected by image analysis). Parallel increases were seen in the percentages of cells immunolabeled for ACTH, indicating that this stimulated population did include corticotropes.

The effects of second messenger activators and AVP were not additive, however indicating that AVP may be working via the same route. Further tests determined if calcium channel blockers would inhibit binding (as they did in a previous study published before the grant was funded (8)). When nimodipine was added with the phorbol ester, the increase in the percentage of ACTH cells was still seen. However, the increment in the percentage of cells that bound CRH was blocked. This indicated selective sensitivity to calcium channel activators and blockers in a subpopulation of corticotropes.

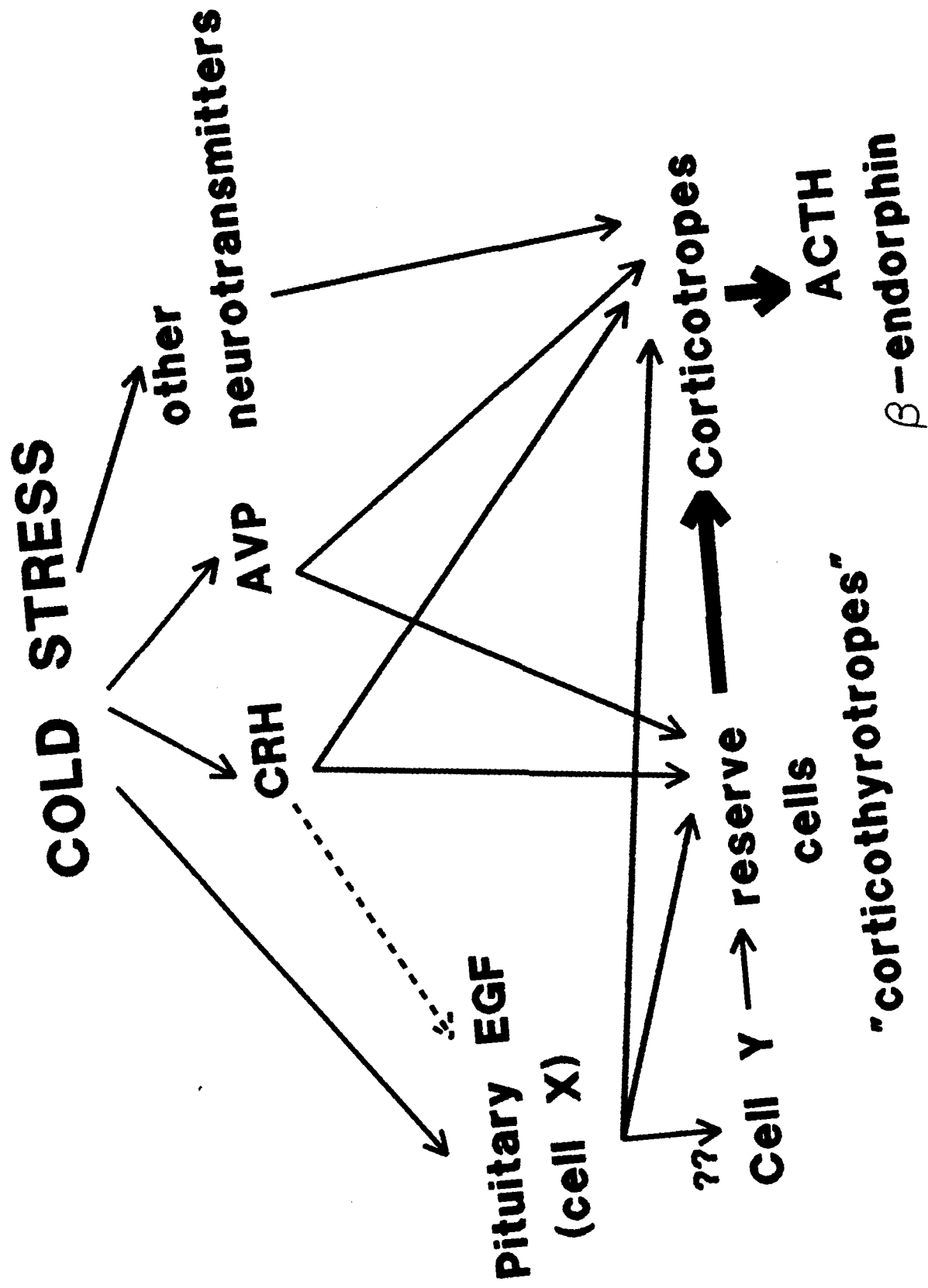
Thus, these studies showed that subpopulations of corticotropes must be activated by AVP (or Angiotensin II, or second messengers) in order to bind CRH. These data fit with earlier data showing that greater numbers of ACTH-secreting cells were seen in the plaque assay if AVP was added with low concentrations of CRH (5). Relating the data to earlier studies of cold stress (4), we postulate that the AVP that is activated from PVN neurons during cold stress serves to stimulate the AVP-dependent subpopulation of corticothyrotropes to produce both ACTH and TSH and bind CRH. Such a cell population would be able to support both the thyroid and adrenal axes in the response to cold exposure.

Phase 7. The effect of negative feedback by corticosterone on AVP or CRH binding by corticotropes. It is well known that corticosterone has a potent negative feedback effect on pituitary corticotropes. Previous studies showed that exposure for 15 h reduced the number of ACTH secreting cells (in a plaque assay) and CRH did not promote recovery of secretion (5). The studies partially supported by NSF DCB-8718242 and this Navy contract explored short term effects of corticosterone on binding to CRH and AVP (9). This was done in part to test the hypothesis that suggested that the reason for the lack of an increase in the amount of label for POMC mRNA in individual anterior lobe cells after 30 (but not 15) min of cold stress was because of the corticosterone feedback encountered *in vivo* (2).

Corticosterone exposure for as little as 10-30 min caused a significant (50%) reduction in the percentage of corticotropes bound by CRH. However, binding by AVP to corticotropes was unaffected (9). Furthermore, when secretion was tested, corticosterone reduced secretion stimulated by biotinylated CRH. A pretreatment for 1 h with AVP was not able to elicit a recovery in the CRH-mediated secretion during the first 30 min of exposure to corticosterone. This indicated that the block affects AVP's ability to stimulate release (although it can still bind to the cells). However, when the cells were pretreated with phorbol esters, recovery of ACTH release was allowed. The CRH binding was still suppressed by corticosterone indicating that activation of protein Kinase C did not promote CRH binding in the face of the steroid. However, the block in secretion was partially alleviated.

These data suggest that a subpopulation of corticotropes are extremely sensitive to corticosterone and although ACTH secretion can be elicited by second messenger activation, they will not bind CRH or respond to AVP. About 40% of corticotropes continue to bind CRH, however, secretion in the population is still reduced so that it is clear the steroid is inhibiting responses. The time frame in this *in vitro* study (10-30 min) highlights the rapid negative feedback actions by corticosterone on responses. It supports the hypothesis that states that the corticosterone blocked expression of POMC mRNA *in vivo* in the group stimulated for 30 min by cold stress. The percentage of corticotropes expressing POMC mRNA was higher than normal, however, individual corticotropes did not express more POMC mRNA than normal (2).

Phase 8. Extension studies: Epidermal growth factor is a mediator of ACTH release during cold stress. The extension of this research grant was originally focused on determining if a shorter period of cold stress would promote increases in POMC mRNA detected in frozen sections. The experiments indicated that differences were still not detected and the problems with the application of the protocol to frozen sections of anterior lobe cells remained unsolved. During the same period, pilot studies were underway to test the effects of epidermal growth factor on ACTH release and expression of POMC mRNA. This growth factor stimulated secretion of ACTH from both mixed and enriched corticotrope cultures indicating that it probably interacted directly with pituitary corticotropes. It also enhanced the stimulatory effects of CRH (10).



The significant interaction of EGF with corticotropes is of particular interest because it is produced by pituitary cells. Therefore, it may be a local mediator of ACTH release. To test this hypothesis, EGF antigens and mRNA were localized in the pituitary in 2-6% of cells plated and fixed within 3 h of removal from the rat (11). We hypothesized that, if EGF was a local mediator in the cold stress response, that exposure might increase expression in the pituitary. Therefore, cells from rats exposed to 30 min of cold or a novel environment were labeled for EGF mRNA or antigens.

There was a significant decrease (from 5.9% to 3.3%) in the percentages of cells labeled for EGF antigens which could have occurred because of secretion. In populations labeled for EGF mRNA, there was a significant increase in rats exposed to cold (from 1.9% to 5.7%) or a novel environment (from 1.9% to 3.3%). Thus, cells that produce EGF are indeed activated by stress. This supports the hypothesis that EGF may serve as a local mediator of corticotrope responses.

SUMMARY AND CONCLUSIONS. These studies have mapped the circuitry of some of hormones activated following cold stress. There appear to be four major hormones that affect corticotropes and thyrotropes during the response to cold exposure. The following figure diagrams the circuitry. CRH's effect may be two-fold, to stimulate release from differentiated corticotropes and to stimulate a subset of reserve cells to bind AVP. In addition, our most recent studies shows that CRH may increase the percentage of cells with EGF mRNA in the pituitary (dotted line). Thus, it may mediate the local control as well.

AVP also increases the potential of the population so that more cells secrete ACTH and bind CRH. Its effect on EGF source cells has not yet been tested. However, it clearly has a stimulatory effect on a population of corticothyrotropes which may comprise the reserve cells with the potential to support both the thyroid and the adrenal. Other neurotransmitters may be involved in this circuitry and they may also activate reserve cells. However, to simplify the diagram, they are listed only on the right hand side.

Finally, EGF stimulates an increase in the percentages of cells that express POMC mRNA. Furthermore, cold exposure increases EGF mRNA. Thus, we postulate that it too is a mediator in the cold stress response and have drawn it into the circuitry. The source of the pituitary EGF remains to be determined however.

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- *3. Childs, G.V. Structure-function correlates in the corticotropes of the anterior pituitary. *Front. Neuroendocrin*, in press, 1992.
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- 5. Childs, G.V. and Burke, J. Use of the reverse hemolytic plaque assay to study the regulation of anterior lobe ACTH secretion by CRF, AVP, A-II and glucocorticoids. *Endocrinology* 120:439-444, 1987.
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- *7. Childs, G.V., and Unabia, G. Activation of protein Kinase C and voltage dependent calcium channels enhances binding of CRH by anterior pituitary cells. *Mol. Endo.* 3:117-126, 1989.
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- *9. Childs, G.V. and Unabia, G. Rapid corticosterone inhibition of CRH binding and ACTH release by enriched populations of corticotropes: Counteractions by AVP and its second messengers. *Endocrinology* 126:1967-1975, 1990.
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- *11. Fan, Xuemo and Childs, G. Cold stress and CRH stimulate expression of epidermal growth factor mRNA in the anterior pituitary. *Proc. 74th Annual Endocrine Society meeting*, San Antonio Tx. June, 1992, p 338

*=Funded by NAVY Contract (see also list of publications at end)

EXPENDITURES.

Supplies: Expenditures normal

Equipment: Equipment was purchased to use true color image analysis. This included a Cue 3 microspectrophotometer, Olympus microscope and cameras, Compaq and Zenith 286 portable (Laptop) computers, and VGA computer monitors to display text and image.

Travel: Each year this grant funded a meeting to present a paper at the Endocrine Society.

Other: Expenditures normal.

PERSONNEL:

Ms Diana Rougeau, Research Technician, 60% support from ONR

Ms. Geda Unabia, Electron Microscopy Technician and Research Assistant II (30% support from ONR).

Dr. Donna Canney, Research Associate, August 1988-January 1989, 100% support from ONR.

Dr. Fumihiko Sasaki, Research Visiting Professor, April 1988-December 1988., 25% support from ONR.

Dr. Ping Wu, Graduate Research Assistant, September 1988-June, 1991. 50% support from ONR

Mr. Xuemo Fan, Graduate Research Assistant, January 1990-present. Support of supplies during extension year, 1990.

TRAINING ACTIVITIES: The following were predoctoral trainees: Dr. Ping Wu and Mr. Xuemo Fan. Dr. Canney was a postdoctoral fellow and Dr. F. Sasaki was a Visiting research scientist in training in techniques of immunocytochemistry. All fellows received training in techniques of handling and care of animals, cell dispersion, culture, immunolabeling, and *in situ* hybridization. Radioimmunoassay techniques were also available to the students.

Women-4; Minorities-3 Oriental students; Non-citizens-3.

Publications: (Peer Reviewed)

1. Childs, G.V., and Unabia, G. Activation of protein Kinase C and voltage dependent calcium channels enhances binding of CRH by anterior pituitary cells. *Mol. Endo.* 3:117-126, 1989.
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(1) (a) Name of Inventor (Last, First, MI) none	(2) (a) Name of Inventor (Last, First, MI) none	(1) Title of Invention none	(2) Foreign Countries of Patent Application none
(b) Name of Employer	(b) Name of Employer		
(c) Address of Employer (Include ZIP Code)	(c) Address of Employer (Include ZIP Code)		

SECTION II - SUBCONTRACTS (Containing a "Patent Rights" clause)

SUBCONTRACTS AWARDED BY CONTRACTOR / SUBCONTRACTOR (If "None," so state)		SUBCONTRACT NO (S)		DAR "PATENT RIGHTS"		DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S)		SUBCONTRACT DATES (YYMMDD)	
a	NAME OF SUBCONTRACT(S)	b	ADDRESS (Include ZIP Code)	c	(1) Clause Number	(2) Date (YYMM)	e	(1) Award	(2) Estimated Completion
	none		none		n/a	n/a	none	n/a	n/a