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Stressor Specificity of Central Neuroendocrine Responses: Implications for Stress-Related Disorders

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Despite the fact that many research articles have been written about stress and stress-related diseases, no scientifically accepted definition of stress exists. Selye introduced and popularized stress as a medical and scientific idea. He did not deny the existence of stressor-specific response patterns; however, he emphasized that such responses did not constitute stress, only the shared nonspecific component. In this review we focus mainly on the similarities and differences between the neuroendocrine responses (especially the sympathoadrenal and the sympathoneuronal systems and the hypothalamo-pituitary-adrenocortical axis) among various stressors and a strategy for testing Selye's doctrine of nonspecificity. In our experiments, we used five different stressors: immobilization, hemorrhage, cold exposure, pain, or hypoglycemia. With the exception of immobilization stress, these stressors also differed in their intensities. Our results showed marked heterogeneity of neuroendocrine responses to various stressors and that each stressor has a neurochemical "signature." By examining changes of Fos immunoreactivity in various brain regions upon exposure to different stressors, we also attempted to map central stressor-specific neuroendocrine pathways. We believe the existence of stressor-specific pathways and circuits is a clear step forward in the study of the pathogenesis of stress-related disorders and their proper treatment. Finally, we define stress as a state of threatened homeostasis (physical or perceived treat to homeostasis). During stress, an adaptive compensatory specific response of the organism is activated to sustain homeostasis. The adaptive response reflects the activation of specific central circuits and is genetically and constitutionally programmed and constantly modulated by environmental factors. (*Endocrine Reviews* 22: 502–548, 2001)

Abbreviations: CART, cocaine- and amphetamine-regulated transcript; DA, dopamine; EPI, epinephrine; GABA, γ-aminobutyric acid; HPA, hypothalamo-pituitary-adrenocortical axis; IML, intermediolateral cell column; NE, norepinephrine; NGFI-B, nerve growth factor, type I-B; NPY, neuropeptide Y; NTS, nucleus of the solitary tract; PVN, hypothalamic paraventricular nucleus.

I. Introduction

HANS SELYE DESERVES much of the credit for introducing the term "stress," and for popularizing the concept of stress in the scientific and medical literature of the 20th century. He wrote in his book, *Stress of Life*: "This lack of distinction between cause and effect was, I suppose, fostered by the fact that when I introduced the word *stress* into medicine in its present meaning, my English was not yet good enough for me to distinguish between the words stress and strain. It was not until several years later that the British Medical Journal called my attention to this fact, by the somewhat sarcastic remark that according to Selye stress is its own cause. Actually I should have called my phenomenon the strain reaction and that which causes it 'stress,' which would parallel the use of these terms in physics. However, by the time that this came to my attention, biological stress in my sense of the word was so generally accepted in various languages that I could not have redefined it. Hence, I was forced to create a neologism and introduce the word *stressor*, for the causative agent, into the English language, retaining *stress* for the resulting condition" (1).

Confusion still arises regarding what one believes defines and constitutes stress. Despite the fact that thousands of research articles have been written about stress and stress-related diseases, until now no scientifically accepted definition of stress exists (2). This results in the view that stress can be practically anything that contributes to virtually any disease in humans. Most scientists view stress as the situation when the hypothalamo-pituitary-adrenocortical (HPA) axis, represented mainly by elevated ACTH levels, is activated (3). Others suggest that activation of other systems with or without an elevation in ACTH may reflect stress-induced disturbed homeostasis (4, 5). Several review articles and book chapters have summarized data from hundreds of stress-related studies and drawn conclusions relating to different aspects of the stress response (2, 6–17). This review focuses on two major points: 1) evidence that specific stressors may elicit specific responses, and 2) different stressors may activate different brain systems by using specific pathways within the central nervous system. Particular attention has been paid to Selye's doctrine of nonspecificity of stress responses, which has been tested in our laboratory. Based on our data using five different acute stressors (immobilization stress, hypoglycemia, nontraumatic hemorrhage, pain stress, and cold stress) and several previous reports by others (4, 18–29), we turned our attention to identifying stressor-specific neuronal circuits in brain and their involvement in stress-related diseases.

II. Stress Concept

A. Definition of stress

Cannon (30–32) was the first to introduce the term "homeostasis" to describe the "coordinated physiological processes which maintain most of the steady states in the organism." He turned his attention to the sympathetic nervous system as an essential homeostatic system that serves to restore stress-induced disturbed homeostasis and to promote survival of the organism. Cannon was also the first to touch on the issue of specificity of stress responses since he showed, for example, that the specific stabilizing or homeostatic reaction to lack of oxygen is quite different from that with which the body responds to exposure to cold; this, in turn, is virtually the reverse of that required to resist heat (1). However, Cannon never used the term "stress."

Selye introduced and popularized stress as a medical and scientific idea. The starting point for the elaboration of his stress theory was his report, published as a letter to *Nature* in 1936 (33), describing a pathological triad (adrenal enlargement, gastrointestinal ulceration, and thymicolymphatic involution) elicited by any of a variety of stressors. From this pathological triad he developed a theory of stress that attained wide popularity and aroused intense research interest but also incited controversy, which persists to the present. He defined stress as the nonspecific response (revealed after subtraction of the specific components from the total response) of the body to any demand, emphasizing that the same pathological triad— "stress syndrome"— would result from exposure to any stressor. According to Selye, these demands on the body included bacterial infection, toxins, x-irradiation, and various physical stimuli such as surgery and muscular exercise. Selye's stress theory did not deny the existence of stressor-specific response patterns; however, he emphasized that such responses did not constitute stress, which was the shared nonspecific component.

Selye mainly focused on the HPA axis as the key effector of the stress response. He considered the adrenal cortex "the organ of integration which participates in the normal and pathological physiology of virtually all tissues in the body," by virtue of its endocrine function (34). Indeed, administration of ACTH can elicit all three components of the pathological triad (34). However, Selye did not assert that HPA activation attending stress reflected the organ pathology in the pathological triad. If anything, Selye asserted the converse.

Selye also introduced the term "general adaptation syndrome" with its three successive phases: the alarm, resistance, and exhaustion stages. He wrote that during the stages of the "general adaptation syndrome" the intensity of the stress response might vary; however, the neural and endocrine patterns characterizing the stage of "alarm" would be essentially the same as those characterizing the other stages. He and others proposed an immense list of diseases of adaptation including hyperfunctional and dysfunctional conditions such as Cushing's disease, hypertension, adrenal tumors, and others. Hypofunctional states included Addison's disease and cancer (1, 2, 6, 7, 17, 34). Later, Selye proposed that most of the stressful stimuli induce two types of responses: 1) a general stress response, which is common to all stressors and involves the release of ACTH and adrenal corticosterone, and 2) individual stress responses mediated by "conditioning factors," such as genetically determined predispositions (17).

In contrast to Selye, Cannon recognized the importance of psychological as opposed to physical responses during stress (30, 35). From an evolutionary perspective he questioned whether a stereotyped response pattern could be adaptive, recognizing that a nonspecific stress response would not have provided an advantage in natural selection and thus, would not have evolved. Others, like Mason, properly noted that in response to different stressors, activity of the HPA axis could increase, decrease, or remain unchanged, implying that the presence of a pathological triad may not indicate the occurrence of stress (2, 13–15). Mason proposed that elicitation of an emotion such as anxiety or fear constituted the basis for similar neuroendocrine responses to different stressors.

Many current views concerning what stress means and how to define and approach it exist, but none has been widely accepted. Many of these theories were discussed in detail and described by Goldstein (Ref. 2 and Table 1). Important contributions to these theories have been made by Weiner (36) and Chrousos (6) and Chrousos and Gold (7). Weiner correctly pointed to specificity of stressor responses by describing stressors as selective pressures from the physical and social environment that threaten or challenge an organism and elicit compensatory response patterns. Chrousos and Gold defined stress as a state of disharmony or of

TABLE 1. Summary of the effects of stressors on 1) Fos immunoreactivity, 2) PVN extracellular NE levels, 3) central nucleus of amygdala extracellular levels, and 4) plasma levels of various hormones

	Stressors				
	Immobilization	Cold	Hypoglycemia	Hemorrhage	Pain
1. *Fos immunoreactivity in various brain regions*					
Hypothalamus					
Paraventricular nucleus	+++	+	+	++	+++
Supraoptic nucleus	++	−	±	++	++
Medial preoptic nucleus	+	+++	−	+	++
Anterior hypothalamic nucleus	+	++	−	−	+
Arcuate nucleus	+	−	+	−	+
Ventromedial nucleus	−	−	−	−	−
Dorsomedial nucleus	++	+	+	+	+
Supramamillary nucleus	++	+	−	−	+++
Lateral hypothalamic area	++	+	++	+	++
Thalamus					
Midline nuclei	++	+	+	+	+++
Limbic system					
Central amygdala	++	−	+	−	++
Lateral septum	++	+	−	−	++
Medial habenula	−	−	−	−	±
Hippocampus	±	−	−	−	±
Cingulate cortex	+++	+	−	+	+++
Piriform cortex	+++	+	−	+	+++
Midbrain					
Substantia nigra	−	−	−	−	−
Central gray	++	±	±	+	++
Dorsal raphe	+	−	−	−	+
Pons					
Parabrachial nuclei	++	+++	−	±	+++
Barrington nucleus	++	+	+	+	+
Pontine nuclei	+++	±	−	−	−
Raphe nuclei	++	+	−	−	+
Cerebellum	−	−	−	−	−
Medulla oblongata					
Spinal trigeminal nucleus	+	++	−	−	++
Peritrigemial nucleus	++	+++	−	−	±
NTS	+	++	+	+	++
Area postrema	−	−	−	++	+
Lateral reticular nucleus	+	−	+	+	++
Catecholaminergic cell groups					
A1	+++	+	−	+	++
A2	++	±	+	+	+
A5	+++	−	−	−	++
A6 (locus coeruleus)	+++	±	−	+	+++
A7	+++	−	−	−	++
A12 (dopamine)	−	−	−	−	−
Spinal cord	++	++			+++
2. *PVN microdialysis*					
NE	+++	+	+	±	++
3. *Central nucleus of amygdala*					
NE	+++	ND	ND	ND	ND
4. *Plasma*					
NE	+++	+++	++	±	+++
EPI	++	±	+++	±	+
ACTH	+++	±	++	+	++
Corticosterone	+++	++	++	+++	+++

+++, High level activity; ++, moderate level activity; +, low level activity; ±, just detectable; −, undetectable. ND, own data not available.

TABLE 2. Stress theories

Author	Definition of stress	Reference
Bernard	Introduced the term *milieu interieur*.	(496)
Cannon	Introduced the term *homeostasis*.	(30)
Selye	Introduced the terms *stress*, *eustress*, *distress*, and *stressor*. Introduced the *general adaptation syndrome*. *Defined stress as the nonspecific response of the body to any demand upon it*.	(1,33,34)
Mason	Criticized Selye's doctrine of nonspecificity. Anxiety and fear understood as the main factors contributing to nonspecific responses upon exposure to various stressors.	(14)
Hennesy and Levine	Introduced a "*psychoendocrine hypothesis*" of stress and arousal.	(497)
Krantz and Lazar	Defined psychological stress in terms of a "*transaction*" between an organism and its environment.	(498)
Munck and Guyre	Incorporated *inhibitory effects of glucocorticoids* in the development of "diseases of adaptation"	(499)
Levine and Ursin	Incorporated *adaptive biological responses* into the definition of stress.	(500)
Weiner	Stress defined as an *external experience or phenomena* to the organism.	(36)
Chrousos and Gold	Defined stress as *a state of disharmony or of threatened homeostasis* evoking both specific and nonspecific responses. Included *genetic polymorphisms* as important determinants of individual stress responses.	(7)
Goldstein	Defined stress as a condition where expectations, whether genetically programmed, established by prior learning, or deduced from circumstances, do not match the current or anticipated perceptions of the internal or external environment. This discrepancy between what is observed or sensed and what is expected or programmed elicits patterned, compensatory responses.	(2)
McEwen	Incorporated the term allostasis as the process of adaptation of the body upon the exposure to various stressors.	(11)

threatened homeostasis, evoking physiologically and behaviorally adaptive responses that can be specific to the stressor or generalized and nonspecific and that usually occur stereotypically, producing a "nonspecific" stress syndrome when the threat to homeostasis exceeds a threshold. They included genetic polymorphisms as well as alterations in the expression of genes and environmental factors as important determinants of individual stress responses.

Based on an intervening variable as a theoretical construct in psychology that links a stimulus to a behavioral response, Goldstein (2) recently introduced a new definition of stress. He defined stress as "a condition where expectations, whether genetically programmed, established by prior learning, or deduced from circumstances, do not match the current or anticipated perceptions of the internal or external environment, and this discrepancy between what is observed or sensed and what is expected or programmed elicits patterned, compensatory responses."

Recently, McEwen (11) introduced the term "allostasis" into stress research. Allostasis, which may be defined as an ability to maintain stability of the internal milieu through change, was originally proposed by Sterling and Eyer (37). As discussed recently in detail by McEwen (11), allostasis refers to the active process of adaptation by productions of various mediators such as adrenal steroids, catecholamines, cytokines, tissue mediators, and immediate early genes. Upon exposure to a chronic stressful situation, physiological responses are initiated, leading to allostatic (adaptive) responses. These responses involve major systems similar to the stress effector systems that were described previously. If allostatic responses are efficient, adaptation occurs and the organism is protected from damage. In situations where allostatic responses are prolonged, inadequate, overstimulated by repeated "hits" from multiple stressors or if a lack of adaptation occurs, allostatic load results in maladaptation and damage to various organs (11, 16). In contrast to homeostatic mechanisms, allostatic regulations are broader and do not depend on set-point mechanisms, signals are not constant, and anticipation of need is an important element. Another aspect of this theory is that allostatic load also reflects aspects of lifestyle (*e.g.*, eating a high-fat diet, lack of exercise, etc.) and disturbances of diurnal rhythms (*e.g.*, sleep deprivation) that result from overexposure of various tissues to stress mediators. Allostatic theory also continues Selye's notion of "conditioning factors" to explain individual differences in stress responses.

Based on our previous findings of the existence of stressor-specific neuroendocrine responses and mapping of stressor-specific central circuits that participate in these responses (see below), we attempted to define stress as a state of threatened homeostasis (physical or perceived treat to homeostasis). During stress, an adaptive compensatory specific response of the organism is activated to sustain homeostasis. The adaptive response reflects the activation of specific central circuits and is genetically and constitutionally programmed and constantly modulated by environmental factors.

Another "mainstream" theory of stress has been offered recently by molecular biologists regarding the role of heat shock proteins in cellular survival (38). Ironically, their theory posits essentially the same doctrine of nonspecificity that Selye espoused; regardless of the insult, cells respond in the same way.

Sapolsky and co-workers (39–41) and McEwen *et al*. (42) introduced and discussed in great detail new aspects of stress in terms of its adverse effects on various brain regions, especially the hippocampus. Upon exposure to stressors, glucocorticoids are released and act on target cells including brain cells. This central action of glucocorticoids is associated with behavioral, neurochemical, and neurodegenerative changes. Neurodegenerative changes are of great importance since they occur in the hippocampus, one of the brain regions involved in memory processes and other cognitive functions as well as in the regulation of the HPA axis (43, 44). Prolonged exposure to high glucocorticoid levels, as commonly seen upon exposure to chronic stress, causes premature age-

related changes in hippocampal electrical activity (45) and dendritic and neuronal atrophy often accelerated upon exposure to neurological insults (*e.g.*, hypoxia) (41). In contrast to these neurodegenerative changes, glucocorticoids also evoke responses that are neuroprotective during exposure to stress (46). For example, various stressors and glucocorticoids increase mRNA expression for oligodendrocyte markers such as glycerol-3-phosphate dehydrogenase and neuronal neurotrophin-3 (46, 47). According to Nichols *et al*. (46), the activation of these markers serves an important adaptive mechanism in promoting oligodendrocyte survival in response to high glucocorticoids levels. In general, glucocorticoids are viewed as key stress hormones that permit, stimulate, or suppress ongoing stress responses, or are preparative during exposure to a subsequent stressor (48).

Oxidative stress is another type of stressor that participates in neurodegeneration of brain cells (49, 50). Expression of mRNA for glial fibrillary acidic protein, an intermediate filament of astrocytes, is increased by oxidative stress, resulting in astrocyte hyperactivation and subsequent damage (49, 50). Recently, CRH and mifepristone, a potent antagonist of glucocorticoid and progesterone receptors, have been shown to protect against neuronal cell death upon exposure to oxidative stress (51, 52). CRH has a neuroprotective action in CRH receptor type 1-expressing neurons against oxidative cell death (52). This CRH protective function is accompanied by increased release of nonamyloidogenic soluble amyloid β-precursor protein and by suppression of nuclear factor-κB. The neuroprotective activity of these drugs may play an important role in new therapeutic interventions for neurodegenerative conditions such as stroke or Alzheimer's disease (51).

B. Classification of stressful stimuli

A stressor may be viewed as a stimulus that disrupts homeostasis. In general, stressors can be divided into four main categories: 1) physical stressors that have either a negative or, in some situations, a positive psychological component; 2) psychological stressors that reflect a learned response to previously experienced adverse conditions; 3) social stressors reflecting disturbed interactions among individuals; and 4) stressors that challenge cardiovascular and metabolic homeostasis (4, 10, 53). Physical stressors include cold, heat, intense radiation, noise, vibration, and many others. Chemical stressors include all poisons. Pain stress may be elicited by many different chemical and physical agents. Psychological stressors profoundly affect emotional processes and may result in behavioral changes such as anxiety, fear, or frustration. Social stressors include an animal's placement into the territory of a dominant animal, and in humans, unemployment and marital separation, among others, are considered social stressors. Stressors that disturb cardiovascular or metabolic homeostasis include exercise, orthostasis, upright tilt, heat exposure, hypoglycemia, and hemorrhage. Many of the stressors described above and used in animal research, however, are mixed and act in concert, such as handling, immobilization stress, anticipation of a painful stimulus, and hypotensive hemorrhage.

In terms of duration, stressors may be divided into two main categories: acute (single, intermittent, and time-limited exposure *vs.* continuous exposure) *vs.* chronic (intermittent and prolonged exposure *vs.* continuous exposure) stressors. It should be noted that many stressors differ in their intensity.

The adaptive responses that are elicited in response to an acute stressor include the physiological and behavioral processes that are essential to reestablish homeostatic balance. During an acute stress response, physiological processes are important to redirect energy utilization among various organs and selectively inhibit or stimulate various organ systems or their components to mobilize energy reserves and to be prepared for exposure to additional, unpredictable challenges. Thus, upon exposure to metabolic stressors, certain tissues tend to reduce their consumption of energy while others, especially those that are important for locomotor activity, receive sufficient nutrients to function properly. The central nervous system also has priority during metabolic stress responses and preferentially receives a sufficient amount of nutrients from the circulation. The increased supply of energy to "crucial" organs is achieved preferentially by release of catecholamines and glucocorticoids that, in general, increase gluconeogenesis and glycogenolysis, inhibit glucose uptake, and enhance proteolysis and lipolysis. The immune system is another essential component of these physiologically adaptive stress responses.

In terms of health consequences during exposure to various stressors, the mechanisms of coping with stress and relevant feedback mechanisms are essential for an organism to develop less severe stress-related health consequences and to survive (54–57). Coping responses during stress may be defined as cognitive and behavioral responses to manage stress (54–58). Cohen and Lazarus (59) defined five primary goals for successful coping with stress: 1) reduce harmful environmental conditions and enhance the prospect for recovery; 2) tolerate or adjust to negative events; 3) maintain a positive self-image; 4) maintain emotional equilibrium; and finally 5) preserve social relationships. There are a number of factors that determine whether an individual will cope effectively with a particular stressor. One of these factors, called the "relevant feedback," is the appropriate feedback from coping responses (55–57). For example, if the relevant feedback to a stressor (unsignaled shock) is low, stress-related symptomatology, *e.g.*, gastric ulceration, increases, while if the relevant feedback is high (signaled shock) less symptomatology is present. Other factors involve appropriate neuroendocrine responses.

The role of neuroendocrine responses in coping with stress is well recognized, since without these responses an organism would be less likely to survive many stressful situations (60). One important feature of successful coping with stress is that physiological systems are not only turned on efficiently by a particular stressor but are also turned off again after a stressor has ceased (60, 61). Thus, when these systems (*e.g.*, neuroendocrine systems) are not rapidly mobilized and then appropriately reduced, elevated hormone levels become dangerous for an organism, resulting in various stress-related diseases (*e.g.*, hypertension, stroke, diabetes, obesity, autoimmune and inflammatory disorders, etc.) (60). The extent to which an individual can cope with stressful situations varies, and these differences are a product of genetics, de-

velopmental influences, experience, training, social support, and current mental and physical health (58, 60, 61).

C. Selye's doctrine of nonspecificity revisited

The parameters of stress observed by Selye were all derived from release of ACTH, which elicits hormonally mediated responses. Stressors, however, also elicit neuronally mediated sympatho-adrenomedullary responses, which although recognized by Selye from Cannon's work, remained unmeasured and therefore were not considered in the syndrome described by Selye (1, 17, 34). Selye described that stressors do not differ in terms of the "patterns" of stress responses. Only after stressor-defined patterns were removed from consideration could one approach the stereotyped stress syndrome. This syndrome could be graded in intensity, but the pattern of response would not be defined by the stressor.

Testing Selye's hypothesis in our laboratory was possible only by comparing the relative magnitudes of several independent neural and hormonal responses at different intensities of stressors using a simplifying assumption: that the magnitudes of both the specific and nonspecific components vary directly with the intensity of the stressor over the whole range of stressor intensities, *i.e.*, that there is no ceiling for the specific component, and no threshold for the nonspecific component (4). Thus, if there is a single unitary response to all stressors, then at two different intensities of the same stressor, the ratios of the increments in the responses should be the same for all parameters, regardless of the stressor. By comparing ratios of differences in response to low- and high-intensity stressors, we examined the theory of nonspecific response patterns.

We measured arterial plasma NE, epinephrine (EPI), and ACTH concentrations in conscious Sprague Dawley rats after exposure to one of five different stressors: immobilization (2 h), hemorrhage (10% or 25% of estimated blood volume; the latter producing hemorrhagic hypotension), cold exposure (4 C or −3 C), pain (evoked by subcutaneous administration of 1% or 4% formalin), or hypoglycemia (evoked by intravenous injected insulin at one of three doses: 0.1, 1.0, or 3.0 IU/kg). For each plasma measure for each animal, an area under the curve (concentration × time) was calculated.

For all three plasma measures, net total responses varied by more than 50-fold across stressors (Fig. 1). At their highest intensity, all of the stressors resulted in significant increases in levels of ACTH, NE, and EPI compared with control values obtained after intravenous saline injection. Immobilization stress evoked large increases in plasma levels of ACTH, NE, and EPI, but other stressors induced disproportionately large NE or EPI responses compared with ACTH responses (Fig. 1). Thus, whereas for ACTH, immobilization stress evoked the largest responses and cold was relatively ineffective, for NE, cold evoked the largest responses, and for EPI, insulin evoked the largest responses. The largest increment in plasma EPI levels after administration of insulin is consistent with the homeostatic effect of EPI in antagonizing the actions of insulin and promoting release of glucose from the liver. Similarly, the largest NE responses after cold are consistent with sympathetic activation to conserve heat by

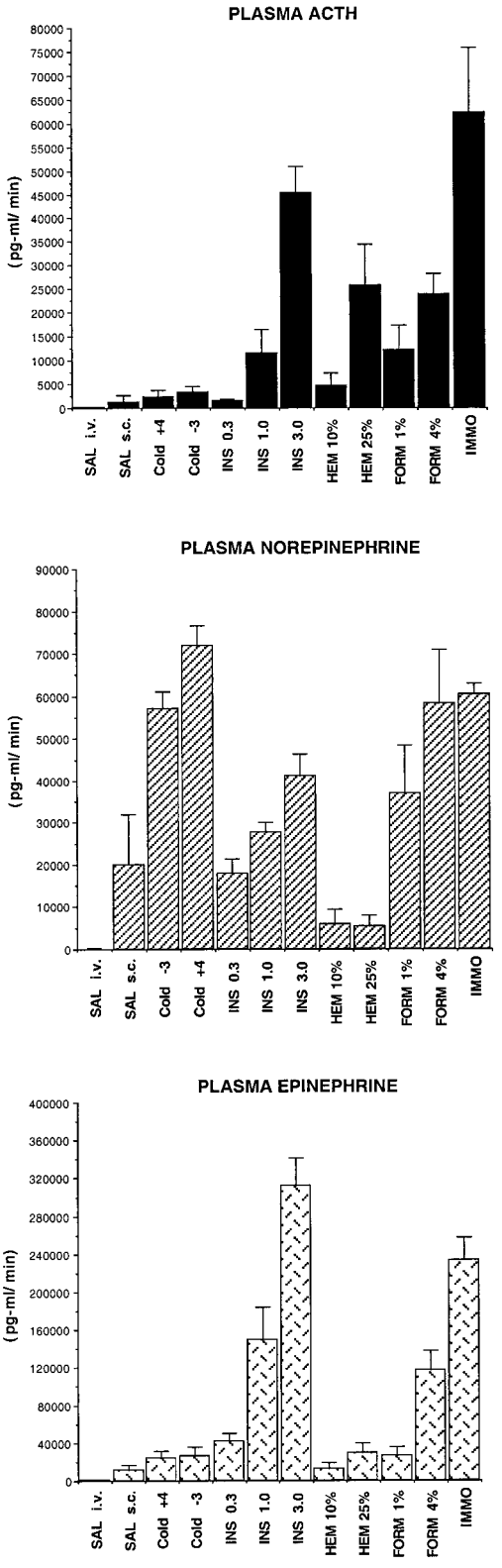

FIG. 1. Responses of plasma ACTH, NE, and EPI during exposure of conscious rats to various stressors. Each *bar* represents the mean value for the net area under the curve (AUC) for that stressor, where net AUC for each animal was calculated from the baseline AUC subtracted from the total AUC. SAL, Saline; INS, insulin; HEM, hemorrhage; FORM, formalin; IMMO, immobilization.

piloerection, vasoconstriction, and energy expenditure. No clear specific responses were found for immobilization stress, hemorrhage, and pain stress; therefore, these stressors were suitable for testing of Selye's doctrine of nonspecificity. Immobilization stress could not be used because the intensity of this stressor could not be varied. Thus, only data for ACTH, NE, and EPI responses to hemorrhage and formalin were appropriate for testing the doctrine of nonspecificity.

The 4% formalin concentration elicited about a 2-fold larger plasma ACTH response and about a 4-fold larger plasma EPI response than did the 1% concentration, and the differences for both variables were statistically significant. Hemorrhage evoked small NE and EPI responses relative to ACTH responses. The 25% hemorrhage elicited about a 5-fold larger ACTH response than did the 10% hemorrhage. There were no differences in plasma NE and EPI responses when the 10% and 25% hemorrhages were compared.

As shown in Fig. 2, for plasma EPI, the ratio of the response for the 25% hemorrhage to the 10% hemorrhage was smaller than the ratio of the response for 4% formalin to 1% formalin. The doctrine of nonspecificity would predict that the difference between hemorrhage and formalin would also obtain for plasma ACTH; in fact, however, for plasma ACTH, the ratio of the response for the 25% hemorrhage to the 10% hemorrhage was much larger than the ratio of 4% formalin to 1% formalin. The increment in plasma EPI levels between the two intensities of formalin was larger than the increment in plasma ACTH levels; yet the increment in plasma EPI levels between the two intensities of hemorrhage was smaller than the increment in plasma ACTH levels. Clearly, the response patterns to these two stressors are not identical.

Our results confirmed the marked heterogeneity of neuroendocrine responses. The present results are consistent with the alternative concept that each stressor has its own neurochemical "signature," with quantitatively, if not qualitatively, distinct central mechanisms. Considering that our studies included assessments of activities of only three peripheral stress effector systems, one would expect that measurements of activities of other systems (*e.g.*, vasopressin, oxytocin, renin-angiotensin, parasympathetic) would yield even more clearly distinct patterns.

The central nervous system plays a crucial role in elicitation and modulation of compensatory stress response patterns. Although a large number of neurotransmitters, neuropeptides, and neuromodulators are activated in various

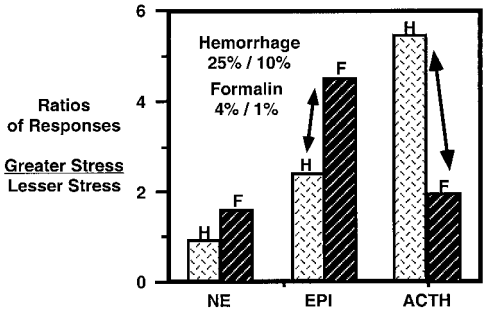

FIG. 2. Ratios of plasma NE, EPI, and ACTH responses to greater or lesser intensities for hemorrhage and formalin. The application of Selye's doctrine of nonspecificity would predict that *arrows* should be parallel to each other and of the same length.

brain regions during exposure to stress, one can predict that specific neuronal circuits exist to optimize effective, rapid, and efficient responses to restore disturbed homeostasis and ensure minimal damage to the organism. This is supported by the elegant work of Gaillet *et al.* (62), who suggested a differential involvement of PVN noradrenergic pathways in the regulation of the HPA axis according to the nature of the stressor. Thus, identification of such "stressor-specific" anatomical and functional circuits would be extremely important in developing future therapies for stress-related disorders. In this review, based on our studies and the work of others, we have attempted to describe stressor-specific anatomical circuits. The mapping of these stressor-specific neuroanatomical circuits is the first step to move the field of stress research in a new direction.

III. Brain Regions Involved in Neuroendocrine Responses to Stress

Although the entire central nervous system is involved in the maintenance of internal homeostasis and participates in the organization of stress responses, some areas may have specific roles in these regulatory mechanisms. They are summarized briefly in this section.

Stressful stimuli may reach the central nervous system through somato- or viscerosensory pathways through spinal or brainstem sensory neurons (Fig. 3). Somatosensory signals are detected by noxious, mechanical, thermosensitive, etc., or specific (photic, acoustic, taste, equilibral) receptors and carried by spinal and cranial sensory nerves. Viscerosensory signals arise from the body and may reach spinal and supraspinal receptors by neural (from interoceptors) or humoral pathways. (Accordingly, stressful stimuli have been previously classified as neurogenic and systemic stressors.)

Stress responses in general can be divided into short and long circuit categories (Fig. 3). Short circuit mechanisms are also called spinal stress responses based on spinal reflexes, while the "long circuits" are also called supraspinal stress responses. The maintenance of homeostasis requires precise coordination of autonomic, neuroendocrine, and behavioral responses to contend with constant perturbations of the internal and external environments. Therefore, the long circuits include higher centers such as the neuroendocrine hypothalamus, the limbic system, and the cerebral cortex. Each of them is neuronally connected with brainstem and spinal somato- and viscerosensory centers, and they are also interconnected with each other (Fig. 3). The output system (*i.e.*, the realization of the stress response) involves two major routes: neuronal and neuroendocrine. The neuronal responses are carried by either somatomotor or visceromotor (autonomic) fibers in cranial or peripheral nerves. Thus, both motor and autonomic stress responses finally arise from brainstem or spinal neurons. The modulatory centers (hypothalamus, limbic system, neocortex) have no direct neuronal outputs to the periphery, but they may exert their effects through actions on brainstem or spinal motor or autonomic neurons (Fig. 3). The hypothalamus has a special neuroendocrine output route, the neurohumoral hypothalamo-pituitary system, which is involved in a prominent fashion in stress responses. Al-

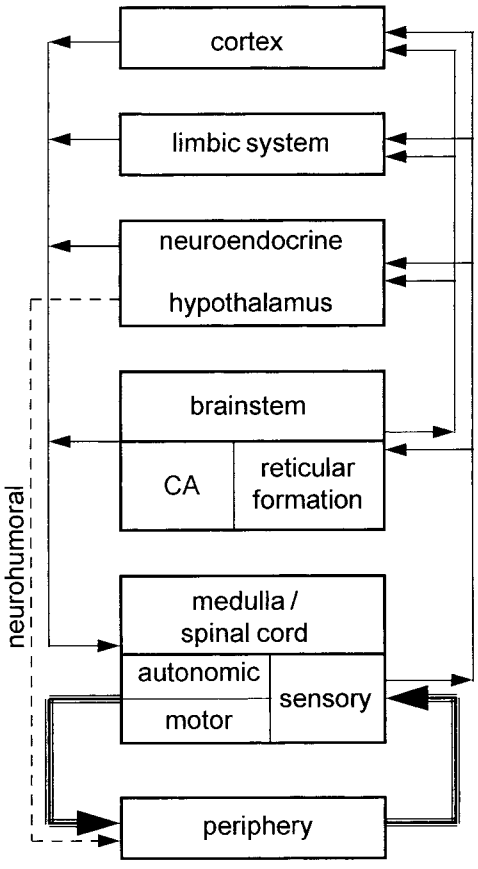

FIG. 3. Neuronal circuits in the organization of stress responses. *Horizontal thick and thin lines* indicate "short circuit": autonomic (sympathoadrenal and/or parasympathetic) and defense (withdrawal) spinal reflexes in response to stressful stimuli. *Thin lines* represent "long circuit": ascending (afferent) and descending (efferent) neuronal loops between the spinal cord/medulla and "higher" brain centers. *Dashed line* indicates neurohumoral hypothalamo-pituitary outflow. CA, Brainstem catecholaminergic neurons.

though the HPA axis is the most representative and probably the most effective neuroendocrine regulatory pathway in the stress response, hypothalamo-pituitary control of other endocrine organs, as well as control of body fluid and mineral homeostasis and food intake, also constitute important regulatory circuits that are involved in the organization of responses to stressful stimuli.

A. Central autonomic system

Preganglionic neuronal cell groups constitute the output in the effector loop of stress responses. These cholinergic neurons in the medulla and the spinal cord are activated during almost all types of stress responses that influence sympathetic or parasympathetic outflows.

The parasympathetic preganglionic neurons are located in the medulla oblongata and (a minor portion) in the sacral spinal cord. In the medulla, they form distinct cell groups (dorsal motor vagal nucleus, superior and inferior salivatory nuclei). In addition, cells are arranged diffusely in the caudal part of the medulla and form a cellular arc between the dorsal vagal and ambiguus nuclei. The sympathetic preganglionic neurons form a longitudinal cell line in the thoracic (and first lumbar) spinal cord at the lateral portion of the gray matter, and this is referred to as the intermediolateral cell column (IML).

Signals to both types of preganglionic neurons arise through two strictly organized projections: short circuit (reflex) and long circuit (modulatory) neurons (Fig. 3). The short circuit afferents to the parasympathetic preganglionic neurons in the dorsal motor vagal nucleus arise from spinal or cranial sensory (both somato- and viscerosensory) neurons.

Some of the sensory signals (like respiratory) reach the preganglionic cell directly (monosynaptic reflex), but the vast majority of the inputs are relayed by sensory neurons in the nucleus of the solitary tract (NTS). The efferent fibers of the short circuit reach the ganglionic cells (in the vegetative ganglia or intramural ganglionic cells) through the vagal nerve. In addition to the inputs to the dorsal motor vagal nucleus, sensory signals ascend from the NTS to brainstem (parabrachial), hypothalamic, and limbic areas constituting the ascending loop of the long circuit (Fig. 3). In addition, noradrenergic and adrenergic neurons within and around the NTS (A2 noradrenergic and C2 adrenergic cell groups, respectively) receive stressful stimuli through sensory pathways innervating the NTS (63). The long circuit afferents to the dorsal motor vagal neurons (descending loop of the circuit) (Fig. 3) arise from the limbic, hypothalamic, and brainstem nuclei, partly directly, partly relayed by neurons in brain regions such as the lateral hypothalamus, the bed nucleus of the stria terminalis, the parabrachial nuclei, and the periaqueductal central gray (64).

The short circuit afferent fibers to the spinal sympathetic preganglionic neurons in the IML arise in dorsal root ganglion cells with a relay by dorsal horn interneurons. Preganglionic efferents leave the spinal cord through the ventral roots and terminate on sympathetic ganglionic cells located either in the peripheral sympathetic ganglia or in the innervated organs (intramural ganglionic cells). Descending fibers to the IML (long loop efferents) (Fig. 3) arise in limbic, hypothalamic, and brainstem nuclei (64).

From a functional point of view, central biogenic amine-containing neurons can be considered as a part of the central autonomic system. While biogenic amines are present in the peripheral autonomic system, central aminergic neurons represent a very specific "one-way" regulatory system. Having neuronal inputs through both somato- and viscerosensory fibers and feedback signals from hypothalamic, limbic, and cortical areas, aminergic neurons are unique projecting neurons with hundreds of axon collaterals and ten of thousands of axon terminals. All of their nerve endings terminate within the central nervous system and none of them project to the periphery. Therefore, their characteristics are briefly summarized in a separate subsection.

B. Central aminergic systems

Brain adrenergic, noradrenergic, and serotonergic neurons are involved in the central processing of stress responses. The role of dopaminergic neurons is controversial in this respect.

Brainstem catecholaminergic neurons receive direct somatosensory input from spinal cord and trigeminal sensory neurons as well as viscerosensory input from the NTS. Their

activation is stressor specific: certain stressors, such as immobilization or pain-related stimuli, activate them rapidly and substantially, while others may have only minor influences.

1. Norepinephrine synthesizing neurons. Neurons in the ventrolateral and the dorsomedial medulla oblongata are the major sources of noradrenergic nerve terminals in the hypothalamus and the limbic system (65–67). In addition to these, noradrenergic cells in the locus coeruleus also contribute to the central organization of the stress response (67, 68). Lesions of brainstem catecholaminergic cell groups or their ascending fibers block or reduce stress-induced changes in the HPA axis (21).

a. The A1 noradrenergic cell group consists of the most caudal noradrenergic cells in the ventrolateral medulla. They are topographically arranged from the level of the medulla -spinal cord junction up to the level of the area postrema. Axons of these cells, comprising the ventral noradrenergic bundle, ascend to the forebrain and innervate mainly hypothalamic and limbic structures. The highest density of noradrenergic terminals is found in the parvocellular subdivision of the PVN that contains the majority of CRH-synthesizing neurons (67, 69).

b. A2 noradrenergic cells are present in the dorsomedial medulla, partly in the nucleus of the solitary tract (NTS), but a number of NE cells are dispersed into the neighboring nuclei. Ascending noradrenergic fibers from this cell group join the ventral noradrenergic bundle and participate in the noradrenergic innervation of the neuroendocrine hypothalamus (65–67, 69).

c. Locus coeruleus neurons increase their activity dramatically in response to certain stressful stimuli (70). The cerebral cortex, the cerebellum, and the basal ganglia are the major targets of these neurons, but they also participate in the noradrenergic innervation of the hypothalamus and the spinal cord (67, 71, 72). The locus coeruleus is involved 1) in the conduction of stress signals to forebrain areas, and 2) in the organization of stress responses.

ad 1) Locus coeruleus neurons receive stress signals through somato- and viscerosensory pathways via the spinoreticulothalamic tract. Noradrenergic fibers from the locus coeruleus innervate almost the entire forebrain including cortical, limbic, and hypothalamic structures.

ad 2) Noradrenergic axons from neurons located in the locus coeruleus and in the subcoeruleus area descend in the pontomedullary reticular formation and the lateral spinal funiculus and innervate the spinal cord. The spinal projection of the locus coeruleus has been demonstrated by retrograde tract tracing (73–75). Large multipolar cells in the ventral part of the locus coeruleus and the subcoeruleus area project to the spinal cord (76). Using a transneuronal viral labeling technique, these neurons have been demonstrated 3 d after injection of pseudorabies virus directly into the lateral-intermediate zones of the thoracic-lumbar spinal cord (Fig. 4).

d. A5 and A7 noradrenergic cell groups are located in the ventrolateral and lateral pons, respectively. Their neurons project to the spinal cord with special high terminal density to the sympathetic preganglionic neurons in the intermediolateral cell column and to the sensory projecting neurons in the dorsal horn (77, 78). A5 neurons receive direct neuronal input from the PVN (79–81).

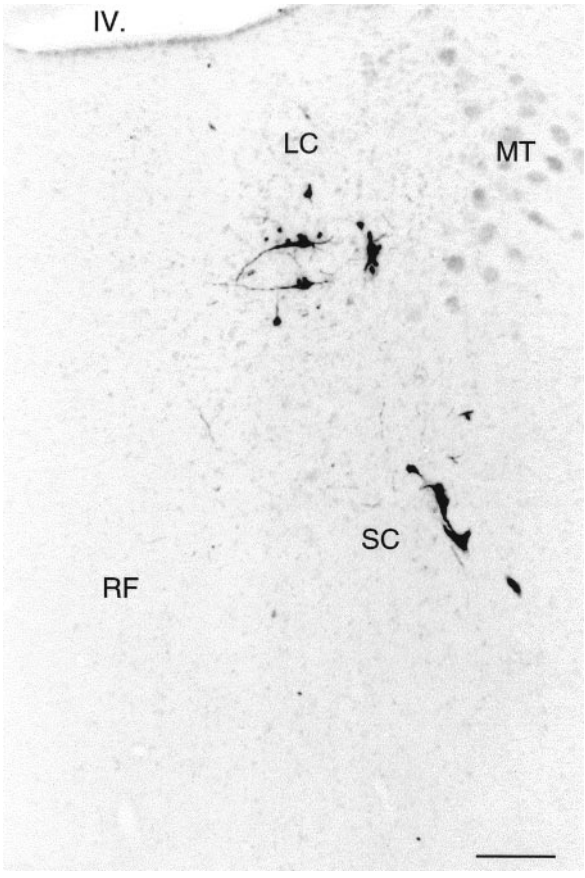

FIG. 4. Retrogradely labeled, pseudorabies-infected cells in the locus coeruleus (ventral portion) and the subcoerulear area 4 d after microinjection of the virus into the thoracic spinal cord (into the intermediolateral cell column and the intermediate zones). LC, Locus coeruleus; MT, mesencephalic trigeminal nucleus; RF, pontine reticular formation; SC, subcoeruleus area; IV, fourth ventricle. *Bar scale*: 100 μm.

2. Epinephrine synthesizing neurons. Adrenergic neurons are present in the middle portion of the ventrolateral medulla (between the A1 and A5 cell groups, rostrocaudally). A separate population of C1 neurons gives rise to a long ascending projection to the endocrine hypothalamus, while others project to the spinal cord to innervate sympathetic preganglionic neurons in the intermediolateral cell column (82–85). The ascending fibers join the ventral noradrenergic bundle. In addition to C1 neurons, adrenergic neurons are also present in the dorsomedial medulla (C2 cell group) just rostral to the A2 noradrenergic cell group. Axons from these adrenergic neurons also join the ventral noradrenergic bundle and participate in the adrenergic innervation of the hypothalamus and the limbic system (84–86).

3. Serotonergic neurons. Serotonergic neurons are found in the lower brainstem (raphe nuclei) and in the hypothalamic dorsomedial nucleus. The rostral raphe nuclei (dorsal, midbrain and linear raphe nuclei) project to the hypothalamus and limbic regions (87, 88), while dorsomedial serotonergic neurons participate in the innervation of the pituitary gland (89, 90).

Serotonergic neurons in the raphe magnus and raphe pallidus (rostral ventromedial medulla) project to the spinal cord (91, 92). TRH-, substance P-, and serotonin-synthesizing cells in the raphe obscurus (and probably in the raphe pallidus) innervate the dorsomedial medulla, including the dorsal motor vagal nucleus and the nucleus of the solitary tract (90).

Serotonergic neurons react sensitively to certain stressful stimuli (restraint, cold, pain) as has been demonstrated by increased c-*fos* activation. Especially, neurons in the raphe pallidus are very sensitive to immobilization stress and formalin-induced pain (refer to *Section V*). Despite a large number of studies, their contribution to the organization of stress responses is still not completely understood.

C. Noncatecholaminergic brainstem neurons

1. Medulla oblongata. The ventrolateral medulla contains stress-sensitive tyrosine hydroxylase-negative neurons. They are present in the lateral reticular and peritrigeminal nuclei. The latter neurons constitute the medullary thermosensitive area and respond to cold stress by rapid c-*fos* activation. In the dorsomedial medulla, NTS neurons are the principal recipients of first-order vagal and glossopharyngeal afferents, which carry viscerosensory signals (baroreceptor, respiratory, gastrointestinal, taste, etc.) to the central nervous system (93). In addition to catecholaminergic neurons (A2 and C2 cell groups), the NTS contains a variety of peptidergic neurons with hypothalamic and limbic projections (94, 95). In addition to these ascending fibers (they form the ascending loop of the "long circuit"), some of the NTS neurons serve as relay neurons that transfer viscerosensory signals directly to brainstem autonomic preganglionic ("short circuit") and catecholaminergic neurons (Fig. 3).

Cells in the ventromedial medulla (serotonin-, TRH- and substance P-containing neurons in the raphe magnus, paragigantocellular, and magnocellular reticular nuclei) project to the spinal cord, both to the dorsal horn and the intermediolateral cell column (92). They may not receive direct nociceptive signals; painful stimuli are carried from the spinal cord by the spinomesencephalic tract initially to the periaqueductal central gray. From here, enkephalin- and dynorphin-synthesizing neurons project down to the ventromedial medulla and disinhibit γ-aminobutyric acid (GABA)ergic interneurons. The activated serotonin-, TRH-, substance P-containing neurons innervate dorsal horn-inhibitory (mainly enkephalin-containing) interneurons that can block or reduce acute pain modulation (96).

2. Pons. Neurons in the parabrachial nuclei (medial, lateral, and Kölliker-Fuse nuclei) may serve important roles as intermediate stations that are modulated by both ascending and descending pathways. The lateral parabrachial nucleus is the main site for the relay of viscerosensory information from the NTS to the forebrain (97–102). The parabrachial nuclei also receive direct neuronal information from the spinal cord and the spinal trigeminal nucleus (98).

3. Midbrain. Cell columns of the periaqueductal gray matter are involved in behavioral, autonomic, and antinociceptive changes. These neurons respond to several stressful stimuli with c-*fos* activation. Considerable data have been accumulated regarding central gray inhibition of pain through the activation of neurons in the rostral ventromedial medulla. Neurons in the lateral and ventrolateral cell columns of the periaqueductal gray matter project to the medullary parasympathetic preganglionic neurons (ambiguus and dorsal motor vagal nuclei) as well as to viscerosensory NTS neurons (103, 104). Other midbrain structures like the colliculi and the geniculate bodies may participate in the organization of responses to specific (optic, audiogenic) stressful stimuli.

D. Thalamus

The midline and intralaminar thalamic nuclei are strongly involved, especially in mammals, in nociceptive mechanisms (refer to the section on Pain for details). Fibers of the spinoreticulothalamic tract terminate here, and nociceptive signals transfer to limbic cortical areas (cingulate, piriform, entorhinal cortex). These neurons influence behavioral responses to certain stressful stimuli. The other sensory thalamic nucleus (ventral posterior thalamic nucleus), which receives nociceptive signals through spinothalamic, trigeminothalamic, and medial lemniscus fibers, represents the subcortical relay center for the discriminative and topographic recognition of sensory signals that terminate in the somatosensory cortex.

Neurons of the midline thalamic nuclei respond to stress with rapid c-*fos* activation (63, 105–108). In various experimental conditions, even the mildest interventions, such as handling and control saline injections, may elicit c-*fos* activation in the midline thalamic nuclei. Thus, Fos positivity in these nuclei after exposure to various stressors should be assessed with caution.

E. Neuroendocrine hypothalamus

Almost all of the medial hypothalamic nuclei participate in the organization of responses to some stressors. The paraventricular, arcuate, and medial preoptic nuclei project to both the median eminence (neurohumoral output) and brainstem/spinal cord autonomic centers (neuronal output). Descending fibers may terminate on autonomic preganglionic neurons directly (64, 109–115) or they may exert their effect through brainstem (A5) catecholaminergic neurons (Fig. 5). Other nuclei, like the ventromedial, dorsomedial, perifornical, and supramamillary nuclei, contain stress-responsive neurons with mainly intrahypothalamic projections. The magnocellular neurosecretory neurons in the paraventricular, supraoptic, and accessory magnocellular nuclei are sensitive to stressors influencing body water and electrolyte homeostasis.

Various inputs mediating stress converge upon neurons of the parvocellular subdivisions of the PVN. These neurons, which synthesize and release CRH and vasopressin (116–118), represent the origin of a final common pathway for neurohormonal regulation of ACTH-corticosterone release.

The lateral hypothalamus may be viewed as comprising a combination of several ascending and descending fibers between the medial hypothalamus, the limbic system, and the autonomic nervous system with thousands of interneurons. Almost all of the stress-conducting fibers enter the hypothalamus in this lateral area. These fibers and a high per-

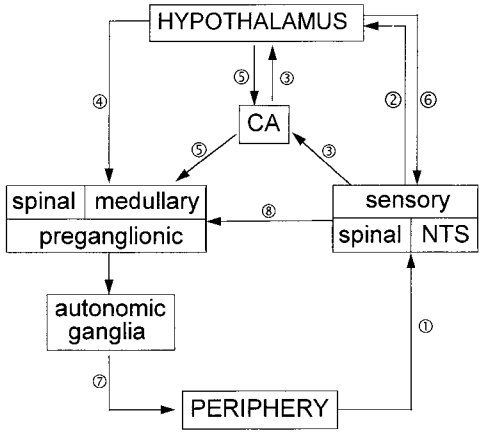

FIG. 5. Stress-related hypothalamo-autonomic neuronal circuits. 1, Stressful stimuli; 2, spinal and medullary viscero- and somatosensory inputs to the hypothalamus; 3, spinal and medullary signals to the hypothalamus through brainstem catecholaminergic pathways; 4, direct hypothalamic projections to spinal and medullary autonomic centers; 5, hypothalamic projections to spinal and medullary autonomic centers through brainstem catecholaminergic neurons; 6, hypothalamic feedback to sensory neurons; 7, stress response to the periphery through the central autonomic system. 1. 8. 7., Short neuronal (periphery-spinal/brain stem-periphery) circuit in response to stressful stimuli.

centage of medial hypothalamic afferent and efferent fibers are relayed here.

F. Limbic system

Both cortical and subcortical limbic structures are involved in the organization of stress responses. The subcortical areas include the amygdala, septum, habenula, and related structures, while the limbic cortex consists of the hippocampal formation (hippocampus, dentate gyrus, subiculum) and entorhinal, piriform, prelimbic, intralimbic, and cingulate cortices (119, 120).

A great variety of behavioral responses to stress are organized by the limbic system. Accordingly, limbic areas *receive* neuronal input from brainstem and spinal viscero- and somatosensory neurons (ascending loop of the "long circuit"), and they *project* to brainstem and spinal autonomic preganglionic neurons (descending loop in the "long circuit"; Fig. 3). With neuronal projections to the hypothalamus, limbic areas may influence the activity of the neuroendocrine hypothalamo-pituitary system (69, 120, 121).

The central nucleus of the amygdala occupies a special position in the organization of stress responses. This nucleus, with four subdivisions, contains various types of peptidergic (CRH, somatostatin, neurotensin, enkephalin, galanin) neurons and receptors (122, 123). They receive brainstem and hypothalamic inputs and project back to these regions directly or through the bed nucleus of the stria terminalis (69, 124). The hypothalamic targets are the PVN and cells within and between the arcuate and ventromedial nuclei (Fig. 6). A significant portion of the brainstem/spinal cord projections of the central amygdala and the bed nucleus of the stria terminalis are relayed by the parabrachial nuclei (Fig. 6).

The septum constitutes an interface between the hippocampus and the hypothalamus. Among other hippocampal neuronal connections, the supramamillary-medial septal-hippocampal afferent projections and the hippocampus-lateral septum-PVN efferent projections are frequently activated by different stressors (69, 120).

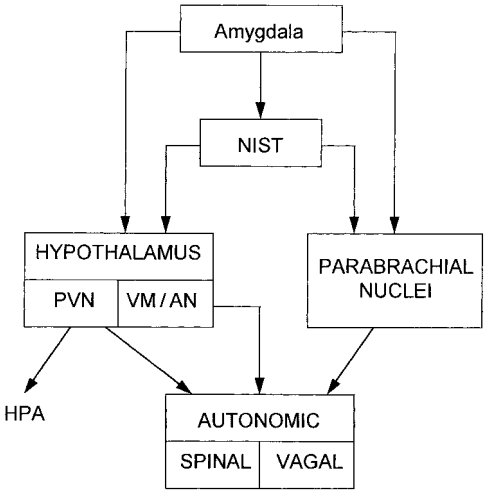

FIG. 6. Projections from the central nucleus of the amygdala to the hypothalamus and to the central autonomic system. AN, Arcuate nucleus; HPA, hypothalamo-pituitary-adrenocortical axis; NIST, bed nucleus of the stria terminalis; VM, ventromedial nucleus.

Limbic cortical regions are sensitive to stress, especially if the stressor exceeds a noxious threshold. These regions are neuronally connected (directly or through entorhinal neurons) with the hippocampus and are responsible for stress-related motivational and behavioral responses (57).

IV. Methods Used for Mapping Stressor-Specific Neuronal Circuits

Several approaches have been introduced to localize and characterize brain structures and neuronal pathways that are involved in the organization of stress responses. Among them, intracerebral microdialysis, immunohistochemistry and *in situ* hybridization for neurotransmitters, neuropeptides and protooncogenes, and tract-tracing techniques are very useful tools, especially when combined with experimental brain surgery (lesioning of brain nuclei, transection of neuronal pathways). Here, we discuss two powerful techniques: intracerebral microdialysis and c-*fos* immunohistochemistry, in detail.

A. Intracerebral microdialysis

Initially, the participation of various brain regions in stress-induced neuroendocrine responses were studied by measuring tissue concentrations of neurotransmitters or related substances. When lower tissue concentrations of a neurocompound of interest were found in a particular brain area, it was assumed that activation of that area had occurred, and the area was considered to be part of a stressor-specific anatomical and functional circuit. However, a comprehensive understanding of neuronal regulation required the development of approaches for assessing simultaneously the rate of delivery of various neurotransmitters into the synaptic cleft

and the magnitude of receptor-mediated postsynaptic responses. It was assumed that a positive correlation would exist, at least in acute stress responses, between neurotransmitter release, its synaptic cleft concentrations, and the activation of an effector system. Much attention was also paid to developing *in vivo* methods that would be applicable in awake animals. Therefore, microdialysis as a new *in vivo* method for manipulating and monitoring neurotransmitter release and inactivation, as well as for evaluating receptor-mediated biochemical effects, was introduced (125–129).

The microdialysis technique makes use of a simple principle. The dialysis membrane is permeable to water and solutes below a certain molecular mass. Perfusion of the microdialysis probe with artificial cerebrospinal fluid or a solution containing a drug of interest creates a concentration gradient across the membrane, causing diffusion of substances across the membrane. In particular, catecholamines, in the extracellular fluid space, diffuse across the dialysis membrane and enter the perfusate, assays of which can then reflect extracellular fluid concentrations of the endogenous compounds. Liquid chromatographic-electrochemical assays of substances in the microdialysate require only small amounts of material, and the samples, under some circumstances, can be assayed directly because the dialysate lacks protein. Microdialysis may also be combined with other techniques such as lesions, local chemical stimulation, pharmacological interventions, and anatomical evaluations, or localized delivery of drugs to specific brain regions, enhancing the value of microdialysis as a tool in experimental neuroendocrinology, neurology, and pharmacology. Our group has applied microdialysis in several studies relating to catecholaminergic innervation of the PVN and stressor-specific NE release in the PVN and its relationship to activation of the HPA axis (24, 25, 130–132). We introduced and described the usefulness of microdialysis in small brain areas such as the central nucleus of the amygdala and the bed nucleus of the stria terminalis (133). We also introduced simultaneous measurements of extracellular fluid levels of NE and its metabolites in various brain regions to provide a comprehensive assessment of synthesis, turnover, release, metabolism, and uptake of monoaminergic neurotransmitters under basal and pathophysiological conditions (24). In all of our studies, we performed our microdialysis experiments no earlier than 20 h after a probe was inserted to minimize factors that could affect neurotransmitter release into extracellular fluid (*e.g.*, surgical stress, acute tissue damage after probe insertion, etc.).

B. Protooncogene-"immediate early genes"-immunohistochemistry

With an ever-expanding knowledge of brain function, attempts have been made to study the activity of individual neuroendocrine cells. Immediate early genes such as c-*fos*, c-*jun*, *jun D*, or *zif268* represent one promising avenue of research. These genes are expressed immediately in response to appropriate extracellular stimuli and then may play important roles in signal transduction and transcriptional regulation in normal cells. Thus, the expression of various immediate early genes in particular neurons is proposed to correlate with their functional activation, and their activation is followed by the production of cell-specific neuroactive substances (28, 28a, 107, 134, 135).

c-*fos* Has been the most frequently used immediate early gene. The pattern of brain activation in response to acute stressful stimuli is examined by using Fos immunohistochemistry (immunostaining the Fos protein product) or *in situ* hybridization (expression of c-*fos*) as markers for neuronal activity. After an appropriate stimulus, c-*fos* expression occurs rapidly, usually within a few minutes, with a peak response within 30 min from the time of the initiation of stress. Fos protein is detectable by immunohistochemistry somewhat later, with maximal levels at 60–90 min after the stressor. The synthesized Fos protein has a half-life of about 2 h.

Initially, it was proposed that responses of immediate early genes to different stressors were rather similar and stereotyped but an increasing number of later studies have clearly demonstrated stressor specificity of c-*fos* responses in different brain regions (for review, see Refs. 19, 28, 107, and 135).

As with other methods, detection of Fos immunoreactivity has some limitations. The identification of Fos in individual neurons can be used for functional anatomical mapping with one caveat, that an absence of c-*fos* induction does not necessarily indicate a lack of neuronal activity (134). The absence of Fos may indicate 1) that a population of neurons does not express c-*fos,* 2) some other immediate early genes and their products are responsible for neuronal stimulation, 3) the signal at the cell body may be insufficient to induce c-*fos* expression, 4) the thresholds for c-*fos* induction may differ in different neurons, or 5) the activating transmitter or the second messenger needed to induce c-*fos* expression was not present, functioned abnormally, or was bypassed (134). In contrast, the appearance of c-*fos* mRNA is not necessarily followed by the production of Fos protein. Another situation in which c-*fos* activation is dissociated from neuronal firing is the presence of a persistent or recurring stimulus. Sustained expression of c-*fos* in response to long-lasting stimuli has been observed in a variety of stressful conditions. Chronic stress may cause persistent stimulation of c-*fos* gene expression, or neurons of certain brain nuclei or regions are activated alternatively. Whether this is due to activation of other intracellular mechanisms or to the intensity of the stimulus remains to be determined.

Recently, NGFI-B (nerve growth factor, type 1-B), also known as nur77, N10, and TISI in mice, has also been used to map neuronal activation at multiple levels of stress-related neuroendocrine circuitry (for review, see Ref. 135). Stressor-specific induction of NGFI-B and c-*fos* expression established NGFI-B as a useful alternative or adjunct marker to c-*fos* for revealing neuronal activation in the neuroendocrine hypothalamus.

V. Stressor Specificity of Central Neuroendocrine Responses

Various stressors elicit different neural and endocrine responses. We analyzed five different stressors under acute

conditions by recording such parameters as plasma ACTH, corticosterone, NE, and EPI levels and extracellular NE levels in the PVN (and in some experiments in limbic nuclei) and investigated c-*fos* activation by Fos immunostaining, and CRH mRNA expression by *in situ* hybridization. Thus, we were able to collect enough information to propose the possible routes and targets of these stressors in the central nervous system. We found that stressors differ not only in their evoked responses but also in their neuronal circuits. It should be pointed out, however, that each stressor may activate several brain structures, and the proposed pathways are the most probable but not the exclusive ones.

A. Immobilization stress

Hans Selye was the first researcher who used immobilization stress, which led in rats to the manifestation of his stress syndrome, *i.e.*, adrenal hypertrophy, gastric ulceration, and thymicolymphatic involution (136). Selye's original restraint procedure involved tying a rat's legs together and wrapping the rat tightly in a towel.

All types of restraint stress should be viewed as a mixture of physical and psychological stressors, including decreased body temperature and pain stress as important components of some restraint procedures. Thus, immobilization stress-induced patterns of activation of various stress effector systems result from restraint, pain stress, and changes in body temperature.

The maximal responses of the stress effector systems are usually seen within the first 30 min after the beginning of immobilization stress. The magnitude of central stress responses usually diminishes upon exposure to chronic intermittent immobilization stress, most likely reflecting habituation as well as exhaustion of stress effector systems. This is well documented by continuous basal or stress-induced decreases in NE release into extracellular fluid in the PVN, most likely due to maximal NE release that is not matched by its ongoing stimulated synthesis in rats exposed to chronic immobilization stress (132, 137).

In our studies, immobilization stress consisted of taping each rat's limbs to a metal frame with each rat kept in a prone position. Our data are in substantial agreement with a number of previous reports of c-*fos* activation in brain nuclei after various types of immobilization or restraint stress.

*1. c-*fos *Expression in the central nervous system after immobilization stress.* Strong c-*fos* activation are observed in several brain regions 30–120 min after immobilization stress, indicating that numerous systems were influenced by this stressor (Table 1).

a. Central catecholaminergic system. Induction of c-*fos* expression in response to immobilization stress is evident within brainstem catecholaminergic cell groups previously shown to play a role in stress-induced activation of the HPA axis (Table 1). Two hours of immobilization stress induces a strong Fos-like immunostaining in the A1, A2, A5, A6 (locus coeruleus), and A7 noradrenergic cell groups (26, 29, 63). Almost all of the tyrosine hydroxylase-positive cells in the ventrolateral medulla and the locus coeruleus are also Fos positive. In contrast to these, only 40% of tyrosine hydroxylase-positive cells (A2 and C2 cell groups) showed Fos immunopositivity in the dorsomedial medulla (Fig. 7). The double-labeled neurons are mainly in the commissural part of the NTS. Tyrosine hydroxylase-positive dopaminergic neurons in the higher and lower brainstem all remain Fos negative after immobilization stress. These included cells in the substantia nigra and the ventral tegmental area (A8, A9, and A10 cell groups), the A11 cell group in the posterior hypothalamus, the A12 cell group in the arcuate nucleus, the A13 cell group in the zona incerta, and the A14 cell group in the preoptic and hypothalamic periventricular nuclei. Acute immobilization stress-induced Fos expression in several brainstem catecholaminergic areas and various brain nuclei has been reported by others as well (19, 28, 106, 107, 138, 139).

b. Brainstem noncatecholaminergic neurons. Several tyrosine hydroxylase-negative neurons in the ventrolateral medulla, some of them in close proximity to the ventral surface of the medulla or the ventral edge of the spinal trigeminal nucleus, are also Fos positive (26, 29, 140). Immobilization stress-induced increases in Fos immunoreactivity in neurons of the spinal trigeminal, peritrigeminal, and raphe nuclei may be related to immobilization stress-induced reductions in body temperature (133, 133a). Almost all neurons in the paratrigeminal nucleus, a cell group that projects to the pontine tegmentum (141, 142), showed Fos immunoreactivity after immobilization stress. Cells in the lateral parabrachial and Kölliker-Fuse nuclei that receive direct neuronal input from the spinal cord and trigeminal nociceptive neurons, but not the NTS (139), show some Fos immunoreactivity after immobilization stress.

Immobilization stress also activates noncatecholaminergic neurons in the dorsomedial medulla (26, 28, 63, 107, 139). A fairly high percentage of tyrosine hydroxylase-negative, neuropetidergic neurons are activated in the NTS after immobilization stress (Fig. 7). These neurons project directly to the hypothalamus and the limbic system (94, 95). Whether Fos immunoreactivity in the paratrigeminal and parabrachial nuclei, including the Kölliker-Fuse nucleus, after immobili-

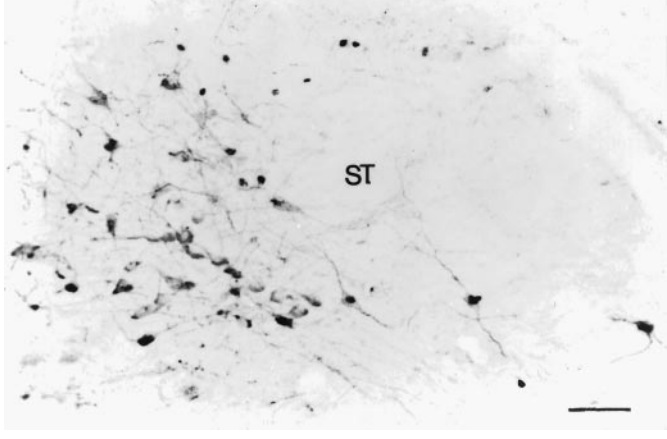

FIG. 7. Fos immunoreactive cell nuclei in tyrosine hydroxylase-positive A2 and tyrosine-hydroxylase-negative neurons in the nucleus of the solitary tract after 3 h of immobilization stress in rats. Note: a number of tyrosine hydroxylase-positive neurons contain Fos-immunonegative nuclei. ST, Solitary tract. *Bar scale*, 50 μm.

zation stress is dependent on somatosensory or unconditioned aversive stimuli is unknown.

c. Thalamus. A widespread pattern of Fos immunoreactivity and c-*fos* mRNA expression (26, 138) are detected in the midline thalamic nuclei 60–120 min after immobilization stress. Like after noxious stimuli (26, 28, 105, 107), the central medial, paraventricular, rhomboid, anterodorsal, reuniens, and intermediodorsal thalamic nuclei, as well as the medial subdivision of the lateral habenula, exhibit marked c-*fos* activation in response to immobilization stress.

d. Hypothalamus. Strong, bilateral Fos immunoreactivity was found in noncatecholaminergic neurons in the hypothalamus after immobilization stress (Table 1). Thirty minutes after immobilization stress, cells in the parvocellular PVN (Fig. 8A) and in the dorsal part of the supraoptic nucleus, most likely oxytocin-containing cells, show c-*fos* activation. However, 2 to 3 h after immobilization stress, pronounced Fos immunoreactivity can be observed in the entire supraoptic nucleus. Thus, it is possible that alterations in salt and water balance as well as changes in blood volume could contribute to these time-related changes in vasopressin-synthesizing supraoptic neurons. Similar to our results, Miyata *et al.* (143) also described increases in Fos immunoreactivity in oxytocin-containing neurons in the PVN and supraoptic nucleus. Cells in the magnocellular subdivision of the PVN and vasopressin-containing cells in the supraoptic nucleus remained Fos negative.

In the parvocellular PVN, Fos-immunopositive cells were distributed through the medial and dorsal subdivisions where the majority of CRH-immunoreactive cells are located (Refs. 19, 28, 107, 138, 144, 145 and Fig. 8A). Two hours of immobilization stress activated c-*fos* in CRH and glucocorticoid receptor-containing PVN neurons (146). The expression was markedly reduced by 120 min after immobilization stress (63). Dopaminergic cells in the PVN appeared to be Fos negative after immobilization stress.

In the preoptic area, dense populations of Fos-positive cells can be located in the ventral and commissural subdivisions of the medial preoptic nucleus but not in its central part or in the lateral and periventricular preoptic areas. In the anterior hypothalamus, Fos-positive cells occupied the ventral subdivision of the anterior hypothalamic nucleus and the lateral hypothalamic area. Fos-immunopositive but not tyrosine hydroxylase-positive cells were seen in the dorsomedial and arcuate nuclei. The ventromedial nucleus and nuclei in the premamillary region were devoid of Fos immunoreactivity (63).

A prominent increase in c-*fos* mRNA expression was detected in the medial parvocellular subdivision and to a much lesser extent in the magnocellular subdivision of the PVN, as well as in the dorsomedial, arcuate, supramamillary, and posterior hypothalamic nuclei, and in the medial preoptic area (138). c-*fos* mRNA levels generally peaked at 30 min after stress, and by 120 min were markedly reduced or had returned to basal levels (138).

e. Limbic system. After three hours of immobilization stress, Fos immunoreactivity was found in the lateral subdivision of the central amygdaloid nucleus and in the medial amygdaloid nucleus. CRH-immunopositive cells in the intermediate subdivision of the central nucleus of the amygdala failed to show c-*fos* activation in response to immobilization stress. Arnold *et al.* (144) and Chen and Herbert (139) did not find c-*fos* activation in the central nucleus of the amygdala after 15–60 min of restraint stress. Similarly, Senba and Ueyama (28) and Arnold *et al.* (144) did not report any changes in c-*fos* activation in the central nucleus of the amygdala after 15–120 min of immobilization stress. Moderate numbers of immobilization stress-induced Fos-immunopositive neurons were demonstrated in the bed nucleus of the stria terminalis, mainly in its dorsolateral subdivision, which contains CRH cell bodies. Strong c-*fos* activation was observed in limbic cortical areas (piriform, cingulate, and entorhinal cortices)

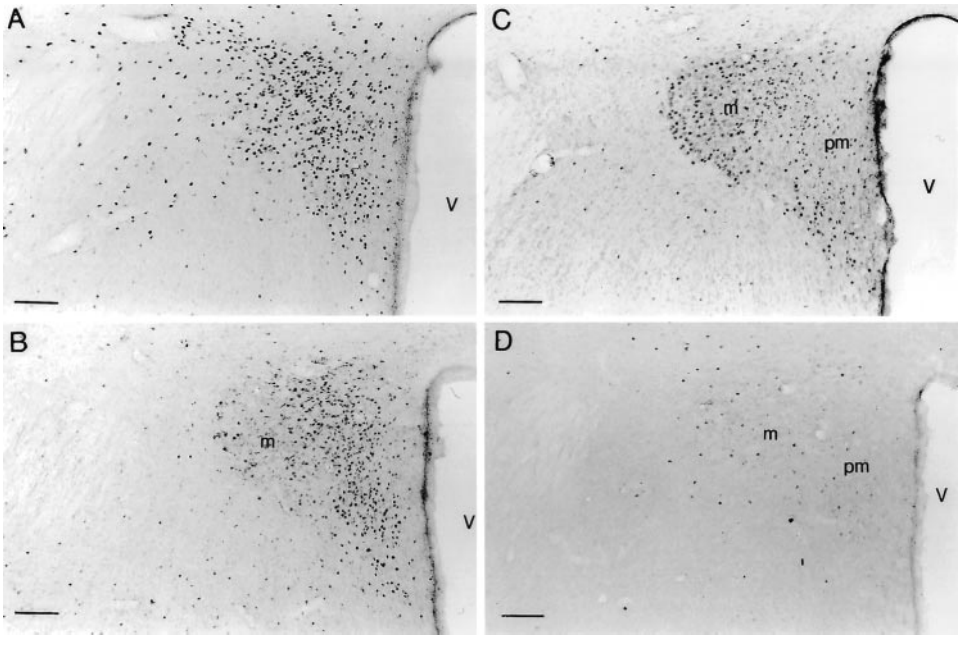

FIG. 8. Fos-immunoreactive cell nuclei of hypothalamic paraventricular neurons. A, After 3 h of immobilization stress; B, 60 min after a 4% formalin injection (subcutaneous) into the hind paw; C, 60 min after withdrawing 25% of the estimated blood volume (hypotensive hemorrhage), and D, 90 min after administration of 3.0 IU/kg insulin (acute hypoglycemia). m, Magnocellular paraventricular subdivision; pm, medial parvocellular paraventricular subdivision; V, third ventricle. *Bar scales,* 100 μm.

after immobilization stress (26, 63). Marked c-*fos* mRNA expression was also detected in the bed nucleus of the stria terminalis, medial and central amygdaloid nuclei, the lateral septal nucleus, medial preoptic area, and the cingulate cortex (138). A marked increase in c-*fos* mRNA expression was detected in the bed nucleus of the stria terminalis, medial and central amygdaloid nuclei, the lateral septal nucleus, and the cingulate cortex 30 min after immobilization stress (138).

f. Cerebral cortex. Neurons in the parietal somatosensory cortex, mainly in layers 3–5, showed c-*fos* activation 30–120 min after immobilization stress (26, 138).

g. Activation of other immediate early genes by immobilization stress. In an extensive study by Cullinan *et al.* (138) c-*jun* and *zif/268* mRNA expression was studied after exposure of rats to restraint stress (30 min in plastic cylinders).

Induction of c-*jun* mRNA after immobilization stress was detected in the NTS, the medial preoptic area, the dorsal premamillary nucleus, the ventral subdivision of the lateral septal nucleus, superficial layers of the parietal cortex, and the infralimbic cortex (138). Prominent expression of transcripts encoding *zif/268* was noted in the parvocellular subdivision of the PVN, the anterior and lateral hypothalamic areas, and the lateral septal nucleus. Moderate increases in *zif/268* mRNA expression were found in the dorsomedial nucleus, the premamillary region, the supramamillary nucleus, and the zona incerta. Relatively minor changes were detected in the brainstem. Thus, as is evident from this study, immobilization stress-induced activation of various brain regions included those regions that participate in the activation of the HPA axis as well as in its inhibition to maintain optimal glucocorticoid levels to "repair" disturbed homeostasis. In terms of HPA axis inhibition, GABA-ergic projections from the anterior hypothalamic area, the medial portions of the bed nucleus of the stria terminalis, and the retrochiasmatic area were proposed to participate in the inhibition of CRH neurons in the PVN (138, 147).

2. Expression of CRH mRNA after immobilization stress.

a. Paraventricular nucleus. We have demonstrated that 3 h of immobilization stress significantly increased CRH mRNA expression in the PVN (148). Bartanusz *et al.* (149) reported that acute immobilization stress increased the levels of both CRH and vasopressin mRNAs in the PVN. Similarly, Kalin and co-workers (150) found that CRH mRNA levels in the PVN were significantly elevated 1 h after cessation of 1 h of restraint. Harbuz and associates (151–153) also demonstrated significant increases in CRH mRNA expression in the PVN 4 and 8 h after exposure to 1 h of immobilization stress. Ceccatelli and Orazzo (18) also examined changes in expression of rat PVN CRH as well as TRH, enkephalin, and neurotensin mRNAs after exposure of animals to 1 h of immobilization stress. There were few significant changes in expression of these mRNAs; enkephalin and neurotensin mRNA expression increased only slightly after immobilization stress. Exposure to acute or chronic immobilization stress was also associated with significant increases in CRH receptor mRNA expression in the parvocellular subdivision of the PVN (154).

It is well established that PVN vasopressin potentiates the ability of CRH to promote ACTH release upon exposure of animals to various stressors. Immobilization stress has been shown to increase vasopressin mRNA expression and vasopressin synthesis in the parvocellular subdivision of the PVN, vasopressin accumulation in the median eminence, and its release into the hypophyseal portal system (149, 155, 156). In contrast, no changes in vasopressin mRNA expression were seen in either the posterior magnocellular subdivision of the PVN or the supraoptic nucleus in response to acute or chronic immobilization stress (149, 155, 156).

b. Amygdala. We also examined the effects of 3 h of immobilization stress on CRH mRNA expression in the central nucleus of the amygdala. Despite marked immobilization stress-induced NE release in the central nucleus of the amygdala, immobilization stress did not alter CRH mRNA expression in this nucleus (148). In contrast to our findings, Kalin *et al.* (150) reported that 1 h of restraint stress increased CRH mRNA expression in the whole amygdala measured 1 h after cessation of restraint stress. Similarly, Mamalaki *et al.* (157) demonstrated increased CRH mRNA expression in the central nucleus of the amygdala immediately after a 2-h period of immobilization stress. The discrepancy between our results and previous reports might be related to the experimental design, the severity of the stressor, or time-dependent changes in immobilization stress-induced CRH mRNA expression.

3. Expression of TRH mRNA after immobilization stress. Acute and chronic immobilization stress exerts inhibitory effects on the hypothalamo-pituitary-thyroid axis, as indicated by decreases in circulating levels of TSH and thyroid hormones in rats (158, 159). Our previous observations that hypothalamic TRH mRNA expression is decreased concomitantly during 2 h of immobilization stress in F344 male rats suggests that this inhibitory effect is in part centrally mediated (160). As investigated further by Cizza *et al.* (161), immobilization stress-induced decreases in TRH mRNA expression could result from stress-induced activation of the NE/CRH system. CRH either directly or via stimulation of somatostatin secretion could inhibit TRH production. In contrast to TRH, after exposure to acute immobilization stress, pituitary TSH mRNA is unchanged in adult rats (161).

Immobilization stress-induced changes in the levels of brain TRH and its receptors were demonstrated by Takayama and co-workers (162). He showed that exposure to 30–180 min of immobilization stress resulted in decreased TRH concentrations in the pons, medulla oblongata, frontal cortex, septum, and amygdala but increased levels in the spinal cord. TRH receptor binding was decreased in the hypothalamus, amygdala, and septum.

4. Concentrations of brain catecholamines after immobilization stress. Acute immobilization stress significantly reduced concentrations of both NE and dopamine (DA) in the arcuate nucleus but not in the supraoptic and ventromedial nuclei and the median eminence. Kvetnansky *et al.* (163) demonstrated the effects of acute (20 min) and chronic immobilization stress (150 min daily for 40 d) on NE levels in additional brain nuclei and regions in rats. Acute immobilization stress was found to decrease tissue NE levels in the PVN,

supraoptic, ventromedial, dorsomedial, and arcuate nuclei. Chronic immobilization stress resulted in significant increases in tissue NE levels in the PVN, supraoptic, and dorsomedial nuclei. DA concentrations were significantly increased only in the PVN and dorsomedial nuclei. In a study conducted by Tanaka *et al.* (164), exposure to 15 or 30 min of immobilization stress did not result in any significant changes in rat tissue NE levels in the whole hypothalamus, amygdala, thalamus, hippocampus, cerebral cortex, pons and medulla oblongata, midbrain, and basal ganglia. In contrast, exposure to immobilization stress lasting from 60 to 120 min resulted in significant decreases in NE levels in the hypothalamus, amygdala, thalamus, hippocampus, and cerebral cortex. In the basal ganglia, tissue NE concentrations were significantly increased while the midbrain, pons, and medulla oblongata failed to show any significant changes.

Hellriegel and D'Mello (165) and Tanaka *et al.* (164, 166) reported time-related changes in immobilization stress-induced enhancement of noradrenergic activity in individual brain regions and their correlation with behavior of rats during immobilization stress. Soon after rats are immobilized, they exhibit struggling, vocalization, urination, and defecation, which could be attributed to activation of the hypothalamus, amygdala, and thalamus, brain regions in which NE responses to immobilization stress are first observed and which could participate in the initial coping responses to the stressor. After vigorous struggling behavior, a period of relative quiescence typically ensues, which could reflect activation of brain centers involved in memory processes (8). Using an immobilization technique that involved wrapping rats in an adhesive band, Lachuer *et al.* (23) demonstrated immobilization stress-induced increases in tissue levels of dihydroxyphenylacetic acid, a DA metabolite, in A1/C1, A2/C2, and A6 (locus coeruleus) catecholaminergic cell groups. Only 5 min of immobilization stress led to immediate increases in dihydroxyphenylacetic acid in all three catecholaminergic cell groups, and maximal increases of dihydroxyphenylacetic acid and plasma corticosterone levels were attained 15 min after the onset of immobilization stress. Return to baseline was achieved within 2 h. Continuous immobilization stress for up to 60 min only slightly increased dihydroxyphenylacetic acid in the A1/C1 and A6 areas while a progressive increase in dihydroxyphenylacetic acid and plasma corticosterone levels were seen over 60 min of immobilization stress. Thus, findings of region-specific immobilization stress-induced noradrenergic activation could be related to different functions mediated by noradrenergic systems in these areas during the course of immobilization stress.

Several research groups investigated the effects of immobilization stress on brain EPI levels. Tissue EPI levels were significantly decreased only after 240 min of immobilization stress in A1/C1, A2/C2, and A6 catecholaminergic cell groups (167).

5. In vivo microdialysis studies. Pacak *et al.* (132) were the first to demonstrate immobilization stress-induced NE increases in the PVN. *In vivo* microdialysis was combined with simultaneous measurement of NE and compounds related to NE turnover and synthesis. Two hours of immobilization stress produced rapid, marked, proportionately similar increases in extracellular fluid NE and dihydroxyphenylglycol levels. The responses of NE were larger, both absolutely and proportionately, in the repeatedly (120 min of immobilization stress for 7 consecutive days) stressed group compared with the singly stressed group. This was because of significantly decreased baseline levels of NE in the repeatedly stressed group. Although absolute and relative responses of dihydroxyphenylacetic acid levels did not differ significantly between the groups, baseline dihydroxyphenylacetic acid levels tended to increase with chronic repetition of immobilization stress, suggesting that tyrosine hydroxylase activity was increased. Extracellular dihydroxyphenylglycol concentration reflects two intraneuronal processes: reuptake of released NE and net leakage of NE from vesicular stores (for review, see Refs. 24 and 132). The relative contribution of these two processes to total dihydroxyphenylglycol release depends on neuronal activity. The similarity of the proportionate increases of NE and dihydroxyphenylglycol, as described above, suggests that the increment in microdialysate dihydroxyphenylglycol was from reuptake of released NE. In brain regions, extracellular dihydroxyphenylacetic acid levels reflect oxidative deamination of axoplasmic DA (for review, see Refs. 24 and 132). Increases in extracellular levels of dihydroxyphenylacetic acid therefore reflect noradrenergic as well as dopaminergic activation. Since stress-induced increases in dihydroxyphenylacetic acid are delayed compared with increases in dihydroxyphenylglycol, stress-induced changes in dihydroxyphenylacetic acid tend to support the view that the increases may reflect increased NE synthesis (132). Thus, our findings that immobilization stress increases release of NE, dihydroxyphenylglycol, and dihydroxyphenylacetic acid suggest that acute and chronic immobilization stress increased NE release, reuptake, and metabolism in the PVN. Moreover, after 6 d of repeated immobilization stress, baseline microdialysate NE concentrations were significantly lower, whereas levels of dihydroxyphenylglycol, the deaminated neuronal metabolite of NE, were unchanged. These findings indicate either that chronic intermittent stress enhances reuptake or diminishes release of NE in the PVN. Since the NE/dihydroxyphenylglycol ratio was lower in chronically immobilized rats, we favor chronic stress-induced increases in NE reuptake as an explanation of the findings.

Tanaka *et al.* (168) and Pacak *et al.* (133) found immobilization stress-induced profound increases in extracellular NE levels in the amygdala of rats. In contrast to the PVN, in the central nucleus of the amygdala, repetitive responses to immobilization stress did not differ from acute responses. However, basal NE turnover was decreased in the central nucleus of the amygdala but tended to increase in the PVN in repeatedly stressed animals, suggesting that chronic stress may differentially affect noradrenergic activity in different brain areas.

Using an *in vivo* microdialysis technique, we also examined the effects of 2 h of immobilization stress on NE release in the bed nucleus of the stria terminalis, a relay station for conveying information from the amygdala to the PVN, the lower brainstem and spinal cord autonomic preganglionic neurons (169–174). Microdialysate levels of NE and its in-

traneuronal metabolite, dihydroxyphenylglycol, were markedly increased above basal levels during the first hour of immobilization stress and decreased gradually thereafter. Levels of dihydroxyphenylacetic acid increased gradually over the entire period of immobilization stress. These findings indicate that in rats, a single bout of immobilization stress results in increased synthesis, release, and reuptake of NE within the bed nucleus of the stria terminalis.

A previously described highly positive correlation between NE release in the PVN and plasma ACTH and corticosterone levels and the existence of noradrenergic synapses on CRH cells in the PVN supported the hypothesis that NE released in the PVN could be stimulatory to CRH neurons (175, 176). A strong positive correlation between NE release in the PVN and plasma ACTH responses during immobilization stress in intact rats demonstrated in our studies (24, 25, 130) further supported a stimulatory role of NE for CRH neurons. To clarify further a role for NE in the control of the HPA axis, we assessed the effects of brainstem hemisections on immobilization stress-induced activation of the HPA axis. Lower brainstem hemisections, which disrupt ascending noradrenergic pathways from A1 and A2 areas to the PVN, decreased microdialysate baseline levels of NE and its metabolites in the ipsilateral PVN and markedly attenuated increments in these levels during immobilization stress (148). We also demonstrated that rats with similar hemisections had lower PVN CRH mRNA expression ipsilateral to the lesion and markedly blunted responses after immobilization stress, compared with values in sham-operated rats (148). Surgical or chemical lesions of the ventral noradrenergic bundle markedly decreased CRH immunoreactivity in the PVN and decreased CRH concentrations in hypophysial portal blood (177, 178).

In conclusion, our findings support the view that CRH mRNA expression in the PVN depends at least partially on ipsilaterally ascending noradrenergic pathways arising from the medulla and that increments in CRH mRNA expression in the PVN during immobilization stress depend on an intact ventral ascending noradrenergic bundle as suggested previously (153, 179–181). However, brainstem hemisections failed to alter plasma ACTH and corticosterone levels either at baseline or in response to immobilization stress. Bilateral and redundant noradrenergic innervation of the PVN as well as disruption of many other stimulatory and inhibitory pathways by "nonselective" surgical hemisection could explain the lack of attenuation of plasma ACTH and corticosterone responses during immobilization stress in lesioned animals.

Using unilateral surgical transections of the stria terminalis, the medial forebrain bundle at the rostral hypothalamic level, or the lower brainstem between the obex and the locus coeruleus, Kiss *et al.* (182) demonstrated the participation of different neuronal pathways in the regulation of basal and immobilization stress-induced expression of CRH and CRH receptor mRNAs in the PVN in rats. Only brainstem hemisections significantly decreased basal and 3 h of immobilization stress-induced CRH mRNA expression in the ipsilateral PVN, suggesting that CRH mRNA levels in the PVN are under tonic stimulatory influences of the lower brainstem. The ability to increase CRH mRNA expression in rats with brainstem hemisections even to a lesser extent than in sham-operated rats further suggests involvement of factors other than NE in the regulation of PVN CRH neurons. Transection of the stria terminalis and the medial forebrain bundle had no effect on basal CRH receptor mRNA levels, but lower brainstem transections decreased significantly basal CRH receptor mRNA levels ipsilateral to the knife cut. None of these lesions affected immobilization stress-induced increases in CRH receptor mRNA expression, suggesting that stress-induced changes in PVN CRH receptor mRNA are mediated either by neural inputs from brain areas other than those investigated or by humoral factors. None of these transections affected vasopressin mRNA expression in the magnocellular PVN. Oxytocin mRNA expression in the caudal magnocellular PVN was significantly decreased only after brainstem hemisections.

6. Proposed existence of neuronal circuits/pathways activated by immobilization stress. Immobilization stress evokes extremely variable endocrine, physiological, and behavioral responses by activating motor, autonomic, and HPA systems. Information from Fos immunohistochemistry after immobilization stress indicates that several neuronal cell groups are involved in this stress response.

Several somatosensory signals arise from mechano- and nociceptive receptors carried by spinal and trigeminal sensory fibers. These first-order neurons (mainly through dorsal horn or NTS interneurons) activate spinal and medullary defense (withdrawal) and autonomic reflexes ("short circuit" mechanism in stress response, Figs. 3 and 5). As a consequence of this "alarm" reaction, the viscerosensory (cardiovascular, respiratory, etc.) system as well as the sympathoadrenal system become activated.

The ascending loop of the "long neuronal circuit" involves several neuronal pathways (Fig. 9). Viscerosensory signals carried by vagal and glossopharyngeal fibers enter the brainstem to synapse in the NTS. Here, these fibers and their collaterals terminate on both the viscerosensory and the catecholaminergic (A2 cells) neurons of the NTS. The viscerosensory NTS neurons relay information bilaterally to the pontine parabrachial nuclei or directly to forebrain (hypothalamic, limbic) structures. The parabrachial nuclei, in turn, relay visceral information to the thalamus, amygdala, and the insular cortex. This pathway appears to contribute to the autonomic organization of visceral responses to stress involving cardiovascular, respiratory, and gastrointestinal activities. Direct NTS-forebrain projections may relay in the lateral hypothalamus to terminate on neuroendocrine (mainly PVN) and limbic (amygdala) neurons (Fig. 9).

Somatosensory signals through first-order sensory neurons enter the dorsal root in the spinal cord (terminating mainly in layers I-V and X of the dorsal horn), while signals from the head and neck regions are carried by the trigeminal, facial, glossopharyngeal, and vagal somatosensory fibers to the spinal trigeminal nucleus. From these primary sensory regions, stress-induced signals transfer through at least four major pathways (Fig. 9).

1. Nociceptive signals to the sensory ventral posterolateral and posteromedial thalamic nuclei are carried by the spinothalamic, trigeminothalamic, and medial lemniscus path-

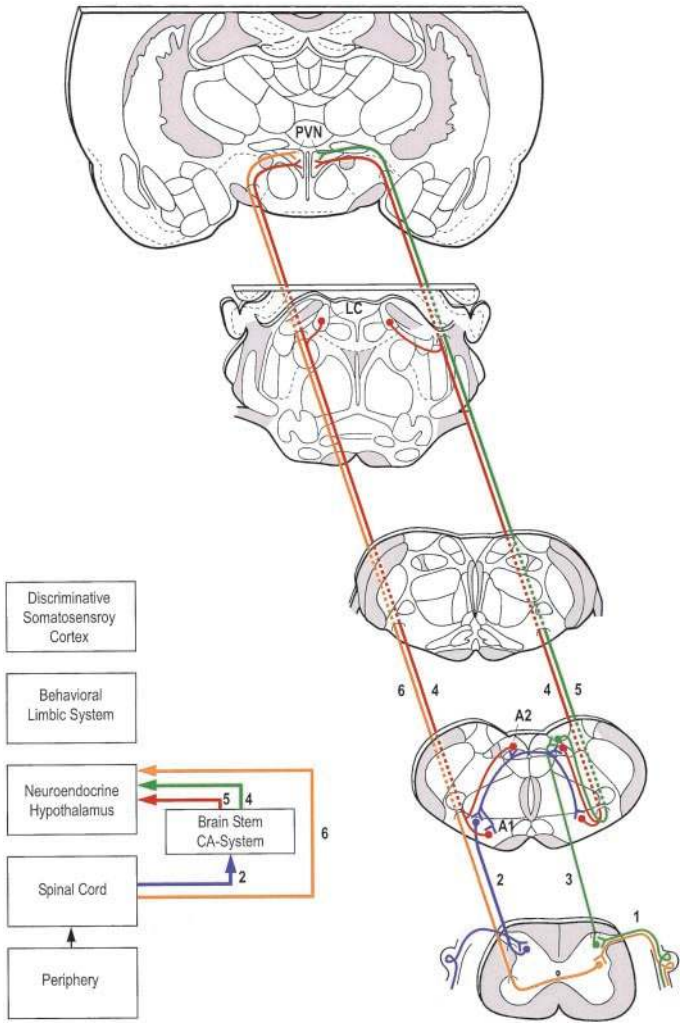

FIG. 9. Neuronal efferent (ascending) pathways to the hypothalamic paraventricular nucleus that are activated by immobilization stress. 1, First-order somatosensory neurons; 2, spinoreticular tract; 3, spinosolitary tract; 4, ventral noradrenergic bundle from A1, A2, and locus coeruleus catecholaminergic neurons; 5, ascending noncatecholaminergic pathway from the nucleus of the solitary tract; 6, spinohypothalamic tract. A1 and A2 represent A1 and A2 noradrenergic cell groups; LC, locus coeruleus.

ways. From here, thalamo-cortical fibers arise and terminate in the somatosensory cortex.

2. Some of the dorsal horn neurons constitute the spinoreticulothalamic tract. This multisynaptic pathway relays in various cell groups of the brainstem reticular formation to organize brainstem stress responses, and finally reach the midline and intralaminar thalamic nuclei. From here, signals run to limbic cortical areas that are involved in the organization of behavioral responses to stressful stimuli.

3. A direct spinohypothalamic pathway has also been described (183–185). Fibers arise in dorsal horn neurons and ascend without synaptic contacts in any brainstem neurons up to the lateral hypothalamus. From here, lateral hypothalamic relay neurons transfer the signals to the medial hypothalamus, mainly to the paraventricular, arcuate and ventromedial nuclei (M. Palkovits, K. Gallatz, A. Bratincsak, and Zs. Toth, unpublished observations).

4. Direct projections have been localized from spinal cord sensory neurons to brainstem catecholaminergic (A1 and A2) cells (spinoreticular pathway). Ascending fibers with bilateral terminations reach the ventrolateral medulla (186–191). Some of the fibers in the medulla project to the A2 cells in the NTS (spinosolitary fibers), which also reach viscerosensory inputs from the vagus and glossopharyngeal nerves (93, 192, 193). It should be noted that these viscerosensory inputs are relayed from the NTS to catecholaminergic and noncatecholaminergic neurons in the ventrolateral medulla (194–197). From A1 and A2 neurons, the ventral noradrenergic bundle arises, and with additional adrenergic fibers from brainstem C1-C2 neurons and some noradrenergic fibers from the subcoeruleus area, ascend to the forebrain and innervate the hypothalamus and the major parts of the limbic system (Fig. 9).

The descending loop of the immobilization stress-evoked stress pathway arises from cortical, limbic, hypothalamic, and some lower brainstem neurons (neuronal efferents), which activate motor and autonomic output systems (Fig. 3). The components of the descending loops are interconnected with each other (cortico-hypothalamic, limbic-hypothalamic, limbic-brainstem, hypothalamo-brainstem fibers) to coordinate responses to stressful stimuli.

Pathways from the hypothalamus project to the median eminence (hypothalamo-hypophysial tract) and the autonomic centers (Fig. 10). The autonomic centers contain mainly fibers that directly innervate brainstem parasympathetic preganglionic and spinal cord sympathetic preganglionic neurons (64, 109–115), and some others that innervate A5 noradrenergic neurons (113).

Immobilization stress also activates the somatomotor system as indicated by strong c-*fos* activation of brainstem and spinal cord motoneurons and in the pontine nuclei (cortico-ponto-cerebellar system). Signal transfer from the somatosensory to the somatomotor system is organized at a neocortical level, while the ventral pallidum occupies a position as an interface between the limbic and the somatomotor systems. It has been hypothesized that the ventral pallidum mediates the flow of motivationally relevant information to motor systems (198, 199).

B. Cold stress

The existence of temperature-sensitive central elements and structures that control body temperature was first reported by Bergmann (200) and Tscheschichin (201). Liebermeister (202) suggested homeostatic mechanisms in temperature control by introducing the concept of a "set-point" for body temperature. An increasing number of rather controversial experiments finally indicated that normal temperature regulation required the integrity of the hypothalamus (for review, see Refs. 203–207). Some other brain areas, such as subthalamic, midbrain, and brainstem regions, were considered to be important structures in thermoregulation as well (for review, see Ref. 208).

During the past two decades, it has been shown that the preoptic region of the hypothalamus is the major organizing center for thermoregulation. A variety of thermoregulatory responses can be elicited by activating a group of medial

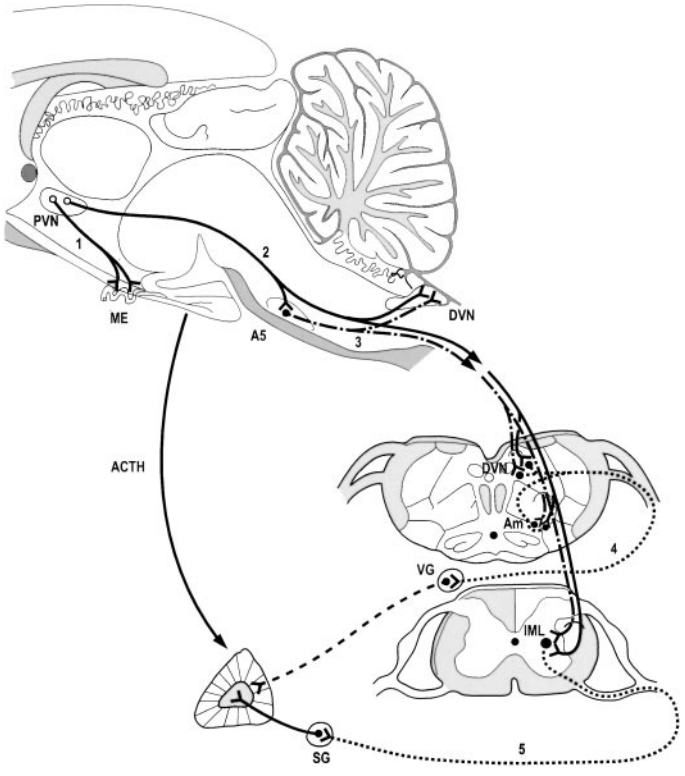

FIG. 10. Neurohormonal and neuronal hypothalamic paraventricular efferent pathways activated by immobilization stress. 1, Hypothalamo-hypophysial tract; 2, descending paraventricular fibers to brainstem and spinal cord autonomic preganglionic, and A5 catecholaminergic neurons; 3, noradrenergic fibers from A5 cells to autonomic neurons; 4, parasympathetic preganglionic fibers from the dorsal vagal and ambiguus nuclei; 5, sympathetic preganglionic fibers from the intermediolateral cell column. A5 represents the A5 noradrenergic cell group; Am, ambiguus nucleus; DVN, dorsal motor nucleus of the vagus; IML, intermediolateral cell column; ME, median eminence; PVN, paraventricular nucleus; SG, suprarenal autonomic ganglia; VG, vagal ganglionic cells.

preoptic neurons (209–225). These neurons receive synaptic input from the periphery through spinal and medullary thermoreceptive pathways. Lipton *et al.* (215) attempted a more detailed mapping of cold-sensitive pathways. Rats in which the preoptic/anterior hypothalamic region was destroyed or disconnected from brainstem structures lost the ability to regulate body temperature properly in response to heat. They demonstrated that 1) communication between the preoptic and anterior hypothalamic (most probably paraventricular) nuclei is required for normal regulation against cold or heat; and 2) a major portion of pathways for regulation against cold passes through the medial forebrain bundle in the lateral hypothalamic area.

1. Cold stress-evoked c-fos expression in brain areas. In our experiments (26, 29, 108, 133a), we exposed rats to cold stress in a temperature-controlled chamber, with temperature maintained at −3 C. Animals were kept in the chamber for 3 h and killed immediately after termination of cold stress. Increased Fos immunoreactivity was found in several brain regions (Table 1). Fos-like immunoreactivity was found in the marginal zone (lamina I) of the dorsal horn, and in the ventrolateral medulla. This group of cells at the ventromedial edge of the spinal trigeminal tract (called the peritrigeminal nucleus) exhibited very strong Fos-like immunoreactivity after cold exposure. Cold stress also activated cells in the paratrigeminal nucleus (a group of cells just dorsal to the spinal trigeminal nucleus). This cell group projects to the pontine tegmentum and especially to the parabrachial nuclei (141, 142), where hundreds of cells showed Fos-like immunoreactivity in response to cold exposure. In noncatecholaminergic cells of the NTS, cold exposure induced Fos-like immunoreactivity in a fairly high number of cells but almost exclusively in the lateral part of the nucleus. Cold exposure elicited mild influences on cells in the raphe nuclei. A small group of cells in the dorsomedial portion of the pontine reticular formation, known as the "pontine thermoregulatory area" in guinea pig (226), also showed Fos-like immunostaining after cold stress. These Fos-immunoreactive cells were located just medial to the subcoeruleus area and ventral to the dorsal tegmental nucleus. Only a very few catecholaminergic neurons in the A1 but not in the A2, A5, A6, and A7 cell groups showed Fos immunoreactivity after cold exposure (up to 10–15% of the tyrosine hydroxylase-positive neurons at maximum). In the hypothalamus, strong c-*fos* expression was found in the preoptic area, especially in the lateral subdivision of the medial preoptic nucleus and in the ventral part of the anterior hypothalamic nucleus (Table 1). Cold exposure had a relatively minor effect on c-*fos* activation in the parvocellular PVN (29).

Miyata *et al.* (227) investigated the effects of acute and chronic cold stress (10 C) on c-*fos* expression in various brain regions. Fos-positive regions were classified into three groups on the basis of the expression period of Fos protein. In the first group, which included the locus coeruleus, the NTS, the dorsal tegmental nucleus, the parvocellular PVN, the posterior hypothalamic area, the supramamillary nucleus, and the lateral septal nucleus, Fos-positive cells were seen 3 h and 24 h after cold stress, but were not observed 14 d after cold stress. The second group consisted of brain areas in which Fos-like immunoreactivity was found 3 h and 1 and 14 d after cold exposure. This group included the medial preoptic nucleus, lateral preoptic area, zona incerta, paraventricular thalamic nucleus, and the lateral parabrachial nucleus. The ventromedial hypothalamic nucleus and the spinal cord were classified into the third group, where Fos immunoreactivity was higher 14 d after cold stress than after 3 h or 24 h of cold stress. In the spinal cord, Fos-positive cells were present in dorsal horn laminae II and III neurons, which participate in thermoregulatory responses such as shivering and vasoconstriction. The time-dependent appearance and disappearance of Fos protein could thus indicate the activation of additional neural pathways participating in homeostasis after exposure to chronic cold stress.

Acute heat exposure induces c-*fos* expression in brain areas in a fashion similar to acute cold exposure (191, 228–232). Heat activates C polymodal nociceptors and heat-sensitive A∂ high threshold nociceptors in the periphery and stimulates laminae I and II neurons in the dorsal horn of the spinal cord (191, 228–230). Among brain regions where cold evoked strong c-*fos* activation, the medial preoptic neurons were very sensitive to heat (231). Thermal stimuli in a noxious range evoked c-*fos* expression in pain-sensitive brain areas

such as the midline thalamic, paraventricular, and supramamillary nuclei (229).

2. Effects of cold stress on the hypothalamo-pituitary-adrenal and other axes. Harbuz and Lightman (152) measured time-dependent responses of CRH mRNA in the PVN and POMC mRNA in the anterior pituitary after exposure to cold stress. Rats were placed in a cold room (4 C) for 1 h, after which they were returned to their vivarium room and killed 1, 2, 4, or 8 h later. Although there were small increases in levels of CRH and POMC mRNAs, especially 4 and 8 h after termination of cold stress, these increments were not significantly different from control animals. In contrast, Zoeller *et al.* (233) found significantly increased expression of CRH mRNA in rat PVN after exposure of rats to acute (6 h) and extended (30 h) periods of cold stress (5 C).

Hauger and co-workers (234, 235) investigated changes in CRH receptors in various brain regions of rats upon exposure to chronic cold stress (60 h at 4 C). Although plasma ACTH levels increased by 3-fold, CRH receptor concentrations in the anterior pituitary were unchanged, whereas in the neurointermediate lobe CRH receptor levels were increased. No changes in CRH receptor levels were demonstrated in the amygdala and the frontal cortex. Immunoreactive CRH content in the intermediate lobe was decreased slightly and increased in the median eminence. Thus, these data support the view that cold stress-induced ACTH release from the anterior pituitary may be at least partly mediated by other mechanisms/factors and pathways than CRH neurons.

Angulo *et al.* (236) studied the effect of cold stress or combined cold and isolation stress (animals housed individually after exposure to cold) on expression of vasopressin mRNA in the paraventricular and supraoptic nuclei. Cold stress (4 C) for 3 h daily for 4 consecutive days increased vasopressin mRNA levels in the posterior magnocellular PVN and had no significant effect on vasopressin mRNA levels in the supraoptic nucleus. In cold-isolated animals, vasopressin mRNA levels were further increased in the posterior magnocellular PVN but not in the supraoptic nucleus. These findings suggest that cold-induced synthesis and subsequent release of vasopressin could have further stimulatory effects on ACTH release at the anterior pituitary level during exposure to cold stress. Furthermore, isolation as a form of stress has been shown to activate the posterior pituitary which, in some clinical settings, could also contribute further to enhanced activity of the HPA axis with some behavioral consequences due to adverse effects of high cortisol levels on brain (11, 237–239).

Acute or chronic cold stress also increases TRH mRNA expression in the PVN, TRH levels in the hypothalamus, and plasma TSH levels, supporting the concept that TRH mRNA levels are reflective of TRH secretion in these neurons (233, 240–243). The effects of cold stress appear to be specific for TRH expression in the PVN, since cold stress does not affect cellular levels of TRH mRNA in other brain regions (233). In support of these findings, Arancibia *et al.* (244–246) demonstrated rapid cold stress-induced TRH release from the median eminence using a push-pull perfusion technique.

Previously, many investigators have reported changes in plasma and brain catecholamine levels upon exposure of experimental animals as well as humans to cold stress (247–256). In our experiments, rats with both an indwelling femoral arterial catheter and with a PVN microdialysis probe were exposed to cold stress at one of two temperatures (4 C and −3 C) (4, 130). Cold stress evoked only very small increments in extracellular PVN NE levels. In plasma, cold stress produced much larger proportionate increments in NE levels than in EPI, ACTH, or corticosterone levels (Fig. 2). These results are consistent with numerous previous reports showing cold stress-induced depletion of hypothalamic catecholamines and selective activation of the peripheral sympathoneuronal system (63). Furthermore, small increments in PVN NE levels and plasma ACTH and corticosterone levels and no correlation between cold stress-induced release of NE in the PVN and activation of the HPA axis suggest that other stress effector systems, such as the hypothalamo-pituitary-thyroid axis, may play a more important role in the maintenance of homeostasis during cold stress. Extracellular serotonin in the PVN was significantly increased in cold-exposed rats (257), suggesting that serotonergic fibers terminating in the PVN may influence TRH synthesis. Serotonin fibers terminating in the PVN arise from the raphe nuclei. Their possible role in body temperature regulation is supported by experimental studies and observations (258–260).

Other stressors, such as acute immobilization, decreased body temperature by 2–4 C, and it remained low for up to 24 h (261). In these animals, brain regions where cold stress was effective (peritrigeminal, paratrigeminal, lateral parabrachial, and medial preoptic nuclei), showed strong Fos immunoreactivity after 2 h of immobilization stress (Table 1) (26).

3. Proposed existence of neuronal circuits/pathways activated by cold stress. Cold stress produces a coordinated response through metabolic, endocrine, autonomic, and behavioral systems. Accordingly, several brain areas and pathways are involved in response to cold-evoked stress. As a working model, we distinguish between extero- and interoceptive thermal stimuli. Temperature changes in the environment activate thermoreceptors. Signals are carried by first-order spinal and cranial nerve fibers, which activate dorsal horn (191, 228) and brainstem (peri- and paratrigeminal) neurons. Thermal stimulation from lamina I and II dorsal horn neurons may reach the ventromedial medulla through the spinoreticular tract, while noxious heat signals are carried by the spinoreticulothalamic tract up to the supramamillary midline and intralaminar thalamic nuclei (229).

Signals from temperature-sensitive interoceptors may be carried by viscerosensory fibers to the NTS (c-*fos* activation in the lateral part of the NTS after cold stress), or influence other thermosensitive neurons through a humoral pathway (by acute changes in blood temperature, which has been observed during certain types of anesthesia or during exposure to subacute stressful stimuli for several hours). It is not yet known whether neurons in brainstem regions where c-*fos* activation was found in cold-stressed animals, *i.e.*, the parabrachial nuclei and the subcoeruleus area, receive first- or second-order thermosensitive signals.

The lateral subdivision of the medial preoptic nucleus and the median preoptic nucleus are considered to be the center

of thermoregulation in the forebrain (227, 230–232, 262–265). These neurons react to thermal stimuli with strong c-*fos* activation. Here, acute cold exposure induces c-*fos* activation in similar fashion as acute heat exposure. Using a combination of tract-tracing and Fos immunohistochemical techniques, projections from lower brainstem areas to the preoptic thermoregulatory cell group were demonstrated in cold-exposed rats (A. Bratincsàk and M. Palkovits, unpublished observations). This pathway (Fig. 11) may represent one of the possible ascending routes for cold-stress signals to the forebrain.

The descending (effector) loop of the cold stress response has not yet been localized. Very little is known about the precise topography of axonal projections of preoptic neurons contributing to thermoregulation. Two proposed pathways should be considered (Fig. 11):

1. The median and medial preoptic nuclei project to the hypothalamic paraventricular nucleus (266). Paraventricular neurons may regulate heat-loss functions through neurohumoral and neuronal mechanisms (Fig. 11): a) Cold stress appears to have no effect on paraventricular CRH (152) but increases TRH mRNA expression (233, 267). Thus, the hypothalamo-pituitary-thyroid axis is one of the candidates for the effector loop of cold-evoked stress responses. b) Preoptic axons terminate on dorsal parvicellular neurons of the paraventricular nucleus, which provide the major descending projections to the sympathetic preganglionic neurons in the intermediolateral cell column of the thoracic spinal cord (266).

2. Neurons in the preoptic area have descending projections to several brain areas. Excitatory signals are conveyed to vasodilatatory neurons in the lateral hypothalamus (268). Preoptic axons terminate in the ventral tegmental area, the periaqueductal central gray, midbrain, pontine, and medullary reticular formation (265, 269). Three further medial preoptic projections have special importance in cold-evoked stress responses (Fig. 11): a) Preoptic axons innervate the rostral medullary reticular formation (269) where parasympathetic preganglionic neurons constitute the superior salivatory nucleus (270). Electrical stimulation of the lateral part of the medial preoptic nucleus produces a marked increase in secretion from the ipsilateral salivary glands. Warming of the preoptic area induces salivary secretion, which is suppressed by cooling of the skin (263). b) Medial preoptic neurons establish bilateral neuronal connections with brainstem serotoninergic raphe nuclei (269, 271), which innervate the spinal cord, including the intermediolateral sympathetic preganglionic cells. Serotonin may play a role in thermoregulation: serotonin synthesis inhibitors completely abolish stress-induced hypothermia (261). c) Preoptic projections terminate in the dorsomedial medulla (266). In conclusion, medial preoptic neurons influence brainstem and spinal autonomic neurons, which regulate the activity of the salivary, thyroid, and adrenal glands, as well as cutaneous blood vessels (Fig. 11), which are significant structures for integrated temperature regulation.

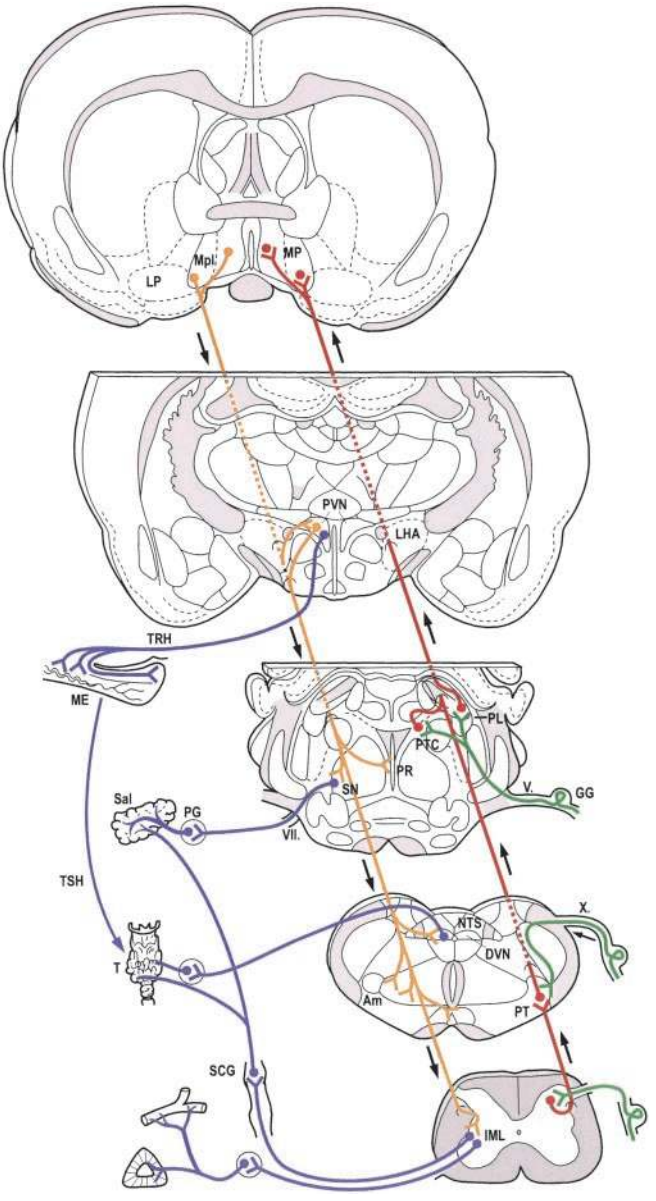

FIG. 11. Proposed thermoregulatory neuronal pathways activated by cold stress. 1, Ascending pathway from medullary and pontine thermosensitive areas to the medial preoptic nucleus (bilateral pathway is shown only on the *right* side) (*green*); 2–6, efferent projections (bilateral pathways are shown only on the *left* side); 2, medial preoptic projections to the PVN (*yellow*); 3, hypothalamo-hypophysial tract (*blue*); 4, medial preoptic projections to brainstem and spinal autonomic centers (*yellow*); 5, Sympathetic (*blue*) and 6, parasympathetic preganglionic fibers (*blue*). Am, Ambiguus nucleus; DVN, dorsal motor nucleus of the vagus; GG, Gasserian ganglion; IML, intermediolateral cell column; LHA, lateral hypothalamic area; LP, lateral preoptic area; ME, median eminence; MP, medial preoptic nucleus; Mpl, lateral subdivision of the medial preoptic nucleus; PG, parasympathetic ganglia; PL, lateral parabrachial nucleus; PR, pontine raphe nucleus; PT, peritrigeminal nucleus; PTC, pontine thermoregulatory center; Sal, salivary glands; SCG, superior cervical ganglion; SN, superior salivatory nucleus; T, thyroid gland; V, roots of the trigeminal nerve; VII, roots of the facial nerve; X, roots of the vagal nerve.

C. Insulin-induced hypoglycemia

Brain function depends mainly on glucose as the major substrate for energy production since, under normal condi-

tions, brain tissue consumes about 50% of the total glucose produced by the liver. Upon exposure to hypoglycemia, neurophysiological and neuropsychological changes occur and develop quite rapidly. They include confusion, abnormal behavior, loss of consciousness, and seizures. Other symptoms such as hunger, sweating, tremor, restlessness, lightheadedness, chills, or sensations of warmth reflect activation of the adrenomedullary system. Together with increased activity of the HPA axis, these stress effector systems increase peripheral plasma glucose levels and delivery of glucose to brain to maintain fundamental brain processes important for an organism's survival (272).

A central neural "glucostat" determines the neuroendocrine responses to glucoprivation, since an intracarotid injection of glucose attenuates, and ganglionic blockade or spinal transection abolishes, the adrenomedullary response to hypoglycemia (273, 274). Although lower brainstem centers can initiate adrenomedullary responses to hypoglycemia, the hypothalamus plays a critical role in hypoglycemia-induced responses of adrenomedullary, sympathoneuronal, and other systems. Cellular glucoprivation induced by local administration of 2-deoxyglucose into the ventromedial hypothalamus evokes rapid and marked increases in plasma levels of glucose, glucagon, NE, and especially EPI (275). Ventromedial hypothalamic lesions attenuate these responses substantially (276), indicating that glucoprivation of cells in the ventromedial hypothalamus triggers glucose counterregulatory responses. As was demonstrated in one of the first "classic" neuroendocrine experiments, hypothalamic paraventricular lesions result in hypoglycemia (277). Nevertheless, identification of neuroanatomical sites and pathways that participate in hypoglycemia-induced responses of various brain regions has not been available until recently, when Fos staining was introduced.

1. Hypoglycemia-induced c-fos expression in brain areas. Hypoglycemia was repeatedly found to induce Fos immunoreactivity in the lateral hypothalamus, reflecting activation of glucose-sensitive neurons located in this area (278, 279), but no Fos immunoreactivity was detected in the ventromedial nucleus, a region containing glucoreceptor neurons (278, 280, 281). Three peptidergic neuronal populations, melanin-concentrating hormone, PRL-like immunoreactive peptide, and orexin synthesizing neurons, have been identified recently in the lateral hypothalamic area and are considered to play an important role in feeding behavior and glucose homeostasis (278, 279, 282–284). A few melanin-concentrating hormone neurons were found to exhibit Fos-like immunoreactivity in contrast to about 80% of PRL-like immunoreactive peptide neurons that expressed c-*fos* after exposure to hypoglycemia. Orexin-containing neurons in the lateral hypothalamus are activated during hypoglycemia. Fos-like immunoreactivity was detected in approximately 33% of orexin-containing neurons in the lateral hypothalamus after a single insulin (20 IU/kg) injection (284). Since insulin receptors are not expressed in the lateral hypothalamus, the activation of these lateral hypothalamic neurons most likely resulted from either local hypoglycemia or activation of some other brain regions that project to the lateral hypothalamus (281). Indeed, it has been established that the lateral hypothalamus is connected with glucose receptor-positive neurons in the medial hypothalamus (ventromedial and dorsomedial nuclei) and other brain regions such as the septum, dentate gyrus, and other areas (285).

Niimi *et al.* (281) evaluated c-*fos* responses to insulin-induced hypoglycemia in other hypothalamic and brain regions. After administration of an insulin dose of 3 IU/kg, blood glucose fell to values less than 40 mg/dl in 3 h when Fos immunoreactivity was most prominent. Acute hypoglycemia induced c-*fos* expression in various hypothalamic nuclei such as the parvocellular division of the PVN, the periventricular, dorsomedial, and arcuate nuclei, and the lateral hypothalamic areas. Only a few Fos-positive cells were detected in the preoptic and anterior hypothalamic areas. Although this study did not examine the colocalization of Fos and CRH, increased Fos immunoreactivity appeared in most of the hypothalamic CRH-producing neurons.

A few cells of the magnocellular division of the PVN and supraoptic nucleus, which contain arginine vasopressin and oxytocin neurons, expressed Fos during hypoglycemia. Similarly, Griffond *et al.* (286) reported that a small number of oxytocin neurons (12–18%) showed Fos immunoreactivity, and these neurons were mainly found in both the parvocellular and magnocellular divisions of the PVN. In the hypothalamic periventricular nucleus, hypoglycemia-induced Fos immunoreactivity corresponded to somatostatin-producing neurons. This is consistent with previous reports in which hypoglycemia was shown to increase hypothalamic somatostatin mRNA levels and to inhibit GH secretion in rats (287). Hypoglycemia also induced increases in Fos immunoreactivity in the bed nucleus of the stria terminalis, septum, amygdala, cerebral cortex, and the thalamic paraventricular nucleus (281). It should be noted, however, that Fos immunohistochemistry is an exceptionally sensitive tool to visualize neuronal activity, and experimental conditions may influence c-*fos* expression nonspecifically. Transfer of animal cages or the animals in a short interval before injection or cannulation and handling of the animals without at least 5–7 d adaptation can result in activated c-*fos* expression in the hypothalamic, midline thalamic, and limbic nuclei.

In our experiments, we exposed rats to acute hypoglycemia using an insulin dose of 3 IU/kg to investigate changes in Fos immunoreactivity in brainstem neurons and other brain regions (26, 29). Rats were killed 90 min after a single insulin injection. Although blood glucose decreased substantially with a nadir of less than 40 mg/dl 30 min after an insulin injection, hypoglycemia had only minor effects on Fos immunoreactivity in tyrosine hydroxylase-positive cells in the A2 area (0–2 cells per section), but not in the A1 cell group or the locus coeruleus. Fos-like immunopositivity was also detected in some of the tyrosine hydroxylase-negative neurons in the dorsomedial medulla (26). In the diencephalon, Fos-like immunoreactivity was found in the zona incerta and the dorsomedial hypothalamic nucleus 90 min after administration of 3.0 IU/kg insulin. In the PVN, Fos-like immunoreactivity was found only in a few dorsal and caudal parvocellular neurons (Fig. 8D), mainly in those that project to brainstem and spinal cord (29). In our conditions, no c-*fos* expression was found in the limbic system in acute hypoglycemia except a well circumscribed area in the ventrolat-

eral part of the central nucleus of the amygdala. None of the known neuropeptides, neurotransmitters, or receptors exhibit such a pattern of distribution. Similar to our results, Porter and Bokil (288) did not find increased c-*fos* expression in brain nuclei 60 min after intracerebroventricular, or 90 min after intravenous insulin infusions under euglycemic conditions. When blood glucose decreased, they found c-*fos* activation in the arcuate nucleus, the NTS, and the ventrolateral medulla, similar to our experiment (26). Marked c-fos activation was found in the hypothalamic paraventricular, arcuate, and ventromedial nuclei after intraperitoneal injections of a bolus of insulin plus glucose (288a).

Insulin-induced hypoglycemia did not activate catecholaminergic neurons in the locus coeruleus; Rusnak *et al.* (289) reported no changes in tyrosine hydroxylase mRNA in the locus coeruleus measured 5 h after single or repeated insulin injections (5 IU/kg), which caused a greater than 50% reduction in plasma glucose levels. As discussed later, these findings are also supported by our previous studies that did not demonstrate any hypoglycemia-induced extracellular NE increments in the PVN (130). Chemical lesions of the ascending noradrenergic bundle did not block insulin-induced activation of ACTH and corticosterone secretions (290). In contrast to insulin, single or repeated 2-deoxyglucose administration increased tyrosine hydroxylase mRNA in the locus coeruleus (289). In contrast to insulin, 2-deoxyglucose causes cellular glucodeprivation, its mechanism of action is different, and blood glucose levels are elevated after its administration. Thus, it cannot be excluded that 2-deoxyglucose acts directly to affect tyrosine hydroxylase gene expression.

2. Effects of hypoglycemia on the hypothalamo-pituitary-adrenocortical axis. In clinical practice, insulin-induced hypoglycemia is used to assess the functional integrity of the HPA axis. Delayed responses of the HPA axis to hypoglycemia suggest that these responses are mediated by neural structures that are sensitive to hypoglycemia and carry information either to CRH neurons or ACTH- or cortisol-producing cells (273, 291). Among central regulators that receive such information, CRH and vasopressin play an important role in hypoglycemia-induced activation of the HPA axis since administration of CRH antibodies reduced ACTH responses to insulin-induced hypoglycemia (27, 292).

Hypoglycemia increases CRH mRNA and *c-fos* mRNA expression in the PVN (293–295), CRH turnover in the median eminence (296), CRH and POMC mRNA levels in the anterior pituitary (292, 297, 298), CRH levels in the hypophysial portal and peripheral blood, and depletion of ACTH content in the anterior pituitary (293, 299, 300). Some of these findings are in contrast to the study by Plotsky (27), who found unchanged CRH concentrations but elevated vasopressin levels in portal plasma during hypoglycemia. Administration of a vasopressin V1 receptor antagonist attenuated hypoglycemia-induced plasma ACTH increments, and intracerebroventricular administration of vasopressin significantly decreased hypophyseal portal plasma concentrations of CRH (27). These results suggest that hypoglycemia-induced ACTH secretion may be mediated by dynamic changes in portal vasopressin concentrations and that CRH has a permissive role in hypoglycemia-induced plasma ACTH responses. Furthermore, these results provide support for the hypothesis of stressor-specific hypophysiotropic coding.

Hypoglycemia-induced ACTH increments seem to follow the early induction of c-*fos* in PVN CRH neurons (295). c-*fos* mRNA levels in the PVN are increased significantly 30 min after a single insulin injection of 5 IU/kg, reach a peak at 60 min, and return to baseline by 120 min (294). c-*jun* mRNA concentrations reach a peak at 90 min and remain elevated 120 min after an insulin injection. CRH mRNA accumulation is unchanged until 30 min after an insulin injection; however, a significant increase is observed at 60 min, reaching a plateau at 90 min (294). Chronic hypoglycemia (blood glucose below 80 mg/dl for 5 d) also provokes marked increases in c-*fos* and CRH mRNA expression in the PVN (295).

To detect neural pathways participating in hypoglycemia-induced activation of the HPA axis, several surgical approaches have been used. Using hypothalamic deafferentation and/or lesions of the medial basal hypothalamus, or hypophysectomy, Kárteszi *et al.* (291) and Ježová *et al.* (301) and Weidenfeld *et al.* (302) demonstrated that hypoglycemia mainly stimulated corticosteroid production through neural pathways to the hypothalamus from an anterolateral direction. These workers (302) investigated insulin-induced activation of the HPA axis in rats with various types of hypothalamic deafferentation and measured responses of plasma ACTH and corticosterone after injection of low (0.04 IU/100 g of body weight) or high (0.2 UI/100 g of body weight) insulin doses. The higher insulin dose reduced serum glucose by approximately 50% and elicited a marked increase in serum ACTH and corticosterone levels. The lower dose of insulin, which reduced serum glucose by approximately 30%, elicited a significant adrenocortical response only in intact rats and rats with posterior hypothalamic deafferentation. They concluded that during mild hypoglycemia, activation of neural pathways impinging upon CRH neurons from a rostral direction is crucial for responses of the HPA axis. In contrast, during severe hypoglycemia, adrenocortical responses are mediated by systemic mechanisms that act directly on the medial basal hypothalamus. In addition to these major pathways, hypoglycemia was found to stimulate corticosterone responses through pathways independent of the medial basal hypothalamus but requiring the pituitary (291, 303). This is well supported by studies of Mezey *et al.* (303), who reported that insulin-induced hypoglycemia stimulated ACTH secretion from stalk-sectioned rats. Thus, it appears that both peripheral (humoral) and central (neuronal) pathways are necessary for activation of the HPA axis, and the severity of hypoglycemia determines which pathways are activated (304).

Insulin-induced hypoglycemia is also a primary stimulus for the secretion of both posterior pituitary hormones, vasopressin, and oxytocin (305–307). Vasopressin synergistically enhances CRH-induced ACTH secretion (308–310). Hypoglycemia does not affect vasopressin mRNA in the whole PVN (298) but selectively activates a vasopressin-containing subset of CRH neurons in the parvocellular subdivision of the nucleus (307). Double-labeling experiments demonstrated that the number of CRH mRNA-containing cell bod-

ies that also contained vasopressin mRNA was doubled during insulin-induced hypoglycemia (311). These data, together with increases in the turnover of vasopressin in the median eminence (296) and vasopressin levels in hypophysial portal blood (293), suggest that increases in plasma vasopressin levels during hypoglycemia are derived from activation of PVN CRH neurons.

Insulin-induced hypoglycemia is also known to inhibit the activity of the hypothalamic GnRH pulse generator (312). There is no direct inhibition of GnRH neurons since these neurons remain capable of releasing their hormone during hypoglycemia when stimulated by *N*-methyl-D,L-aspartate (313). Recent studies in which the suppression of GnRH neurons caused by insulin-induced hypoglycemia was blocked by a CRH antagonist or a CRH inhibitor, together with observations that CRH synapses on these neurons, suggest that CRH is an important intermediate involved in this inhibition (314–316).

Orexins are a family of recently identified neuropeptides that have been implicated in feeding behavior: food deprivation for 48 h was shown to up-regulate prepro-orexin mRNA levels in rat hypothalamus (282). They stimulate food intake after injection into the paraventricular, dorsomedial, and perifornical nuclei, or into the lateral hypothalamus (283). Orexin-synthesizing neurons in the lateral hypothalamus project to the arcuate nucleus to innervate neuropeptide Y (NPY)-containing cells, which contain leptin receptors receptors (317, 318). These findings suggest that orexins may play an important role in stress-related changes in feeding behavior.

Among several other factors, the adipocyte-derived hormone leptin (319) is strongly involved in both pathways (320–326). Insulin is thought to stimulate leptin secretion. Increased plasma leptin concentrations are found in hyperinsulinemia, and insulin-induced hypoglycemia is associated with a decrease in leptin concentrations (321, 323–325). The cocaine- and amphetamine-regulated transcript (CART) peptide could be an endogenous factor in brain mediating the effects of stress on appetite (327). Food intake increases CART mRNA expression and, in turn, CART reduces food intake significantly (323, 328). At the hypothalamic level (especially in the arcuate nucleus), this activity of CART neurons is closely associated with the action of leptin and NPY. NPY is a potent stimulator of food intake and regulates feeding behavior (318). Food deprivation increases the activity of arcuate NPY neurons that project to the PVN: Leptin decreases NPY synthesis in arcuate neurons and reduces NPY release in the PVN (326). Taken together, these findings suggest that insulin-induced hypoglycemia may influence the insulin-leptin-CART-NPY circuit. In addition to this mechanism, the existence of a lateral hypothalamic-arcuate-PVN-orexin-NPY circuit has also been proposed in the central control of food intake (329).

Selective adrenomedullary activation during hypoglycemia constitutes key evidence for the differential regulation of sympathoneuronal and adrenomedullary outflow during exposure to different stressors (4, 330). Despite profound adrenomedullary stimulation during systemic glucoprivation, microdialysate concentrations of NE in the PVN increase relatively little (130). During hypoglycemia, increments in plasma ACTH levels relative to those of NE in the PVN exceed increases during responses to other stressors, *e.g.*, pain stress (4, 130). These and other findings led to the concept that in response to stressors requiring metabolic demands without involving marked discomfort or distress, ACTH release does not require a mechanism involving major activation of catecholaminergic pathways terminating in the PVN. In contrast, glucoprivation-induced suppression of LH secretion does appear to depend on NE release in the PVN (331).

3. Proposed existence of neuronal circuits/pathways activated by insulin-induced hypoglycemia. Insulin-evoked hypoglycemia may induce stress responses in two major pathways: 1) neuronal and humoral activation of the HPA axis, and 2) activation of the central autonomic system.

a. Activation of the HPA axis. The activation of the HPA axis in insulin-induced acute hypoglycemia has been discussed in detail in *Section V.C.2*. Both CRH and vasopressin are involved in rapid ACTH release in response to insulin-induced acute hypoglycemia (27, 296, 298, 300, 307, 332). Inputs to hypothalamic CRH and vasopressin neurons that are influenced by blood glucose and insulin levels may arise from the dorsomedial medulla. From the periphery, glucose-sensitive fibers run in the vagus nerve and terminate in the NTS. Insulin exerts direct effects on neurons in the area postrema and the NTS (333, 334). Indeed, hypothalamic lesion-induced hyperinsulinemia can be blocked by vagotomy (335). Although several neuronal pathways from the dorsomedial medulla to the PVN have been reported, the route of information regarding blood glucose or insulin levels from the medulla to the hypothalamus has not yet been localized. It is more likely that neither catecholamines nor serotonin are involved in this neuronal circuit: chemical lesions of ascending NE or serotonin bundles did not block insulin-induced activation of ACTH and corticosterone (290, 336). Lesions of the lateral hypothalamus did not inhibit a full response to insulin-induced hypoglycemia (302).

b. Activation of the central autonomic system. Descending hypothalamic and limbic axons terminate on medullary parasympathetic and spinal sympathetic neurons. These neurons provide preganglionic fibers to peripheral ganglionic cells that innervate the adrenal gland, as well as the pancreas. Cells in the dorsal motor vagal nucleus are the site of origin for parasympathetic innervation of insulin-producing cells in the endocrine pancreas (Fig. 12).

1. From the hypothalamus, the PVN provides a major descending pathway to the intermediolateral cell column and the dorsal motor vagal nucleus of the vagus (69, 109, 110, 115, 116, 288a, 337–340) innervating, among others, neurons with pancreatic projections (103, 341). This pathway consists of a variety of peptidergic axons. At least eight possible neuropeptides (oxytocin, vasopressin, met-enkephalin, CRH, somatostatin, angiotensin II, neurotensin, TRH) have been identified in PVN-spinal/vagal neurons (67, 69, 112, 342).

2. ter Horst and Luiten (343) suggested that hypothalamic dorsomedial neurons provide a substantial direct innervation of vagal-pancreatic neurons. In our studies, immuno-

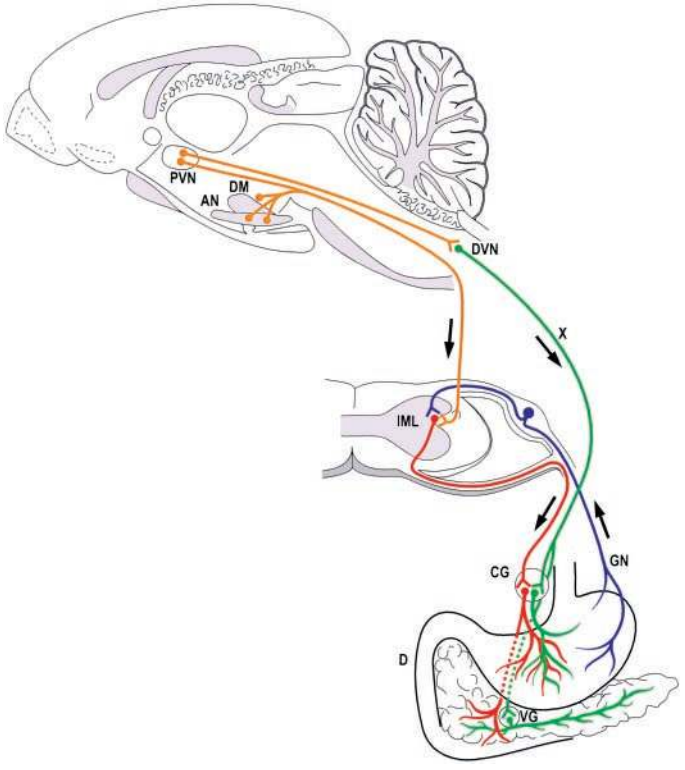

FIG. 12. Proposed neuronal pathways involved in the response to experimental hypoglycemia by a single insulin injection. Hypothalamic projections to autonomic centers, and autonomic innervation of the pancreas. AN, Arcuate nucleus; CG, celiac ganglion; D, duodenum; DM, dorsomedial nucleus; DVN, dorsal motor nucleus of the vagus; GN, sensory gastric fibers; IML, intermediolateral cell column; VG, vagal ganglionic cells; X, vagus nerve.

histochemistry revealed that insulin evokes *c-fos* activation in the dorsomedial nucleus (Table 1).

3. POMC neurons in the arcuate neurons project to the dorsal motor vagal and ambiguus nucleus (344) and thoracic sympathetic preganglionic neurons (320). Some of the arcuate POMC neurons contain CART peptide and are targets of leptin (320). These POMC/CART neurons project to the spinal cord innervating sympathetic preganglionic neurons. This pathway may contribute to the influence of leptin on the autonomic nervous system (320). A fall in leptin concentrations after insulin-induced hypoglycemia correlates with an increase in plasma catecholamine concentrations (321, 324, 325). Insulin itself may not have a direct action on arcuate POMC/CART neurons since an acute injection of insulin did not result in c-*fos* activation in the arcuate nucleus (26, 29, 288) and did not induce POMC mRNA expression (297). A direct action of insulin on arcuate neurons was unlikely since insulin receptors have not been visualized in this nucleus (318).

4. Autonomic preganglionic neurons receive direct input from two limbic forebrain regions, the central nucleus of the amygdala and the bed nucleus of the stria terminalis (119, 122, 123, 170, 341). Insulin-induced c-*fos* activation in a specific group of central amygdaloid neurons suggests that these cells may contribute to acute hypoglycemic stress.

D. Hemorrhage

Depending upon whether hypotensive or nonhypotensive hemorrhage occurs, various physiological responses are elicited upon activation of peripheral mechanoreceptors that are sensitive to volume and pressure alterations. Hemorrhage-induced responses include activation of four main systems, sympathoadrenal, HPA, renin-angiotensin, and hypothalamic atrial natriuretic hormone-vasopressin systems. These responses are important counter-regulatory mechanisms to maintain homeostasis during hypovolemia and may differ due to a variety of factors, including the magnitude and rate of blood loss, starvation, prehemorrhagic hypoxia, or prior exposure to another stressor (345–350).

In experimental and clinical studies, acute hypotensive hemorrhage (usually more than 20% of blood volume is lost) is an extremely dangerous situation since it may lead to shock, a situation in which practically all stress effector systems are activated. If severe hypovolemia is present for a longer time, death is inevitable. Clinical symptoms of nonhypotensive hemorrhage include a low energy level, fatigue, a feeling of being cold, dizziness, and sleepiness; clinical signs of hypotensive hemorrhage include cool or mottled extremities, increased heart rate, low blood pressure, pallor, and altered mental status, ranging from restlessness and agitation to coma. Restoration of blood volume is a high priority, although there are some reports that fluid replacement that is too rapid may be harmful since it may shut down the level of activity of the sympathoadrenal system in the critical period when survival depends on the activity of this stress effector system.

Karplus and Kreidl (351) were the first to implicate the hypothalamus in central autonomic and endocrine regulation of blood pressure. Later, the anterior hypothalamus was found to play an important role in depressor responses, whereas the posterior hypothalamus was found to be involved in pressor responses (352, 353).

Neurons situated in brainstem and pontine catecholaminergic cell groups also appear to be involved in the control of cardiovascular and neuroendocrine function upon exposure to hemorrhage (347, 354–358). Brainstem neurons relay baroreceptor information to other brain regions such as the hypothalamus, brainstem, and spinal cord. Using several experimental approaches including trans-synaptic tracing, antidromic mapping, and c-*fos* expression, findings point to the NTS as the principal site of termination of cardiovascular mechano- and chemoreceptor afferents (for review, see Ref. 354). Most NTS neurons activated upon exposure to hemorrhage are nonaminergic and project to the hypothalamus, the bed nucleus of the stria terminalis, the amygdala, and the ventrolateral medulla (94, 354, 359), where volume- and pressure-related information is interconnected and where regulation of neuroendocrine brain regions occurs directly or indirectly.

Pontine regions, like the locus coeruleus and the parabrachial nuclei, receive cardiovascular information most likely from the NTS via the ventrolateral medulla (for a review, see Ref. 354) and relays information to higher brain regions. Bilateral electrolytic lesions in the anterior portion of the locus coeruleus do not affect blood pressure during nonhy-

potensive and hypotensive (20% of total blood volume) hemorrhage (355). However, animals with lesions in the posterior part of the locus coeruleus show a greater decrease in blood pressure during the hypotensive hemorrhage. The decrease in blood pressure is similar in sham-operated and lesioned animals exposed to severe (30 or 40% of total blood volume) hemorrhage, suggesting that other brain regions and pathways are involved to compensate for the lack of pressor actions of the locus coeruleus (355).

Brainstem and pontine aminergic neurons control the activity of spinal sympathetic preganglionic neurons and are involved in the regulation of neuroendocrine systems, especially the HPA axis (85, 360–362). Posterior hypothalamic knife cuts that disrupt fibers of the medial forebrain bundle at the level of the mamillary body or 6-hydroxydopamine lesions of the dorsal, but not ventral, noradrenergic bundle significantly decrease hypotensive hemorrhage-induced responses of the sympatho-adrenomedullary system, ACTH, renin, and vasopressin (363–365).

1. Hemorrhage-induced c-fos expression in brain areas. In our laboratory (26, 29), we exposed rats to hypotensive hemorrhage by withdrawing 25% of their estimated blood volume through a femoral vein cannula. Rats were killed 60 min after hemorrhage. Hemorrhage activated Fos in a small number of catecholaminergic and noncatecholaminergic neurons in the A1 and A2 cell groups and the locus coeruleus (Table1). In the PVN, hypotensive hemorrhage induced marked *c-fos* expression in the magnocellular subdivision and had only minor effects on *c-fos* activation in the parvocellular subdivision (Ref. 29 and Fig. 8C). Hemorrhage also had minor effects on c-*fos* activity in the supraoptic nucleus. Most of the neurons in the reticular formation, pontine nuclei, and raphe nuclei did not show any Fos immunoreactivity after exposure to hemorrhage (Table 1).

Several other laboratories have used immunocytochemical labeling of Fos protein to determine the effect of hemorrhage on activation of different brain regions, but especially those previously considered to be involved in cardiovascular responses to this stressor. Hypotension caused by hemorrhage or nitroprusside administration results in an increase in Fos immunoreactivity in the locus coeruleus (354, 366). More detailed studies of anatomical distribution showed that after hypotensive (20% of total blood volume) hemorrhage, increased Fos immunoreactivity was detected mainly in the posterior region of the locus coeruleus, suggesting a possible participation of this brain area in blood pressure control (355). Badoer *et al.* (367) examined whether neurons in the rostral ventrolateral medulla that project to the upper thoracic spinal cord and the NTS express c-*fos* after either nonhypotensive or hypotensive hemorrhage. In rats exposed to hypotensive hemorrhage, large numbers of Fos immunoreactive cells were detected in the ventrolateral medulla, the NTS, and the area postrema. In the ventrolateral medulla, Fos immunoreactive cells were present across all rostrocaudal levels and coincided with the region occupied by spinally projecting neurons with presumed vasomotor function (82, 83, 368, 369). In rats with nonhypotensive hemorrhage, the number of Fos-positive cell nuclei was clearly reduced and did not differ consistently from that observed in control rats. In the rostral ventrolateral medulla of rats exposed to hypotensive hemorrhage, the proportion of spinally projecting neurons expressing Fos was significantly greater compared with rats exposed to nonhypotensive hemorrhage or to controls. The proportion of retrogradely labeled cells in the rostral ventrolateral medulla expressing Fos after nonhypotensive hemorrhage did not differ significantly from controls. Chan and Sawchenko (354) calculated that after hypotensive hemorrhage, the proportion of Fos immunoreactive catecholaminergic neurons in A2 and C2 areas that projected to the PVN was 4.5% and 19.3%, respectively. In contrast, more substantial proportions of Fos immunoreactive catecholaminergic neurons in A1 (29.6%) and C1 (32.0%) areas projected to the PVN. About 14.5% and 6.9% of Fos immunoreactive catecholaminergic neurons from the locus coeruleus and A5 cell group, respectively, projected to the PVN.

Both the parvocellular and magnocellular subdivisions of the PVN also exhibit increases in Fos immunoreactivity after hypotensive or nonhypotensive hemorrhage (135, 367, 370, 371). Hypotensive hemorrhage was attended by a significant increase in Fos immunoreactivity in magnocellular neurosecretory neurons in the PVN (including the anterior, medial, and posterior subnuclei). Among the parvocellular neurons of the PVN, dense concentrations of Fos immunoreactive cells were observed in the medial subnucleus, a region known to project to the brainstem and to thoracolumbar spinal cord sympathetic preganglionic vasomotor neurons. Fos-positive cell nuclei were also found in all other parvocellular subnuclei. During nonhypotensive hemorrhage, Fos immunoreactivity increased in the magnocellular and parvocellular subdivisions of the PVN, including the ventromedial subdivision known to contain neuronal projections to brainstem autonomic centers and spinal cord sympathetic preganglionic neurons. Both the parvocellular and magnocellular subdivisions of the PVN also exhibited increased Fos immunoreactivity during isovolemic hypotension (366).

Combined immuno- and hybridization histochemical approaches were used to determine the effect of hypotensive hemorrhage on expression of NGFI-B and c-*fos* mRNAs and Fos immunoreactivity in the endocrine hypothalamus, especially the PVN (135). Hypotensive hemorrhage resulted in expression of NGFI-B mRNA in the dorsomedial part of the parvocellular subdivision of the PVN, a region in which CRH neurons are located. In the magnocellular subdivision of the PVN, hemorrhage-induced expression of NGFI-B mRNA was uniformly distributed across areas containing vasopressin and oxytocin neurons. Hemorrhage also increased NGFI-B mRNA expression in the supraoptic nucleus. Hemorrhage-induced changes in Fos-immunoreactivity and *c*-fos mRNA expression in individual paraventricular subdivisions and the supraoptic nucleus followed closely that of NGFI-B mRNA. Quantitative assessments indicated that 92–98% of all Fos immunoreactive neurons also displayed a positive hybridization signal for NGFI-B mRNA.

By combining tract-tracing techniques with Fos immunohistochemistry, Badoer *et al.* (367, 370) determined whether PVN neurons that project to the rostral ventrolateral medulla, the nucleus of the solitary tract, and the upper thoracic spinal cord and spinally projecting neurons of the rostral ventrolateral medulla express Fos after nonhypotensive or

hypotensive hemorrhage. Retrogradely labeled neurons in the PVN that expressed Fos after hemorrhage were present in all parvocellular subnuclei. The proportion of retrogradely labeled neurons expressing Fos was highest in rats with hypotensive hemorrhage and lower, but still significantly different, from controls in rats with nonhypotensive hemorrhage. Similar to the rostral ventrolateral medulla where only 22% of spinally projecting neurons expressed Fos after hypotensive hemorrhage, 14% of spinally projecting neurons in the PVN expressed Fos after hypotensive hemorrhage. Only a small (5%) subpopulation of PVN neurons projecting to the rostral ventrolateral medulla was activated by hypotensive hemorrhage. Furthermore, Chan and Sawchenko (354) demonstrated that with the exception of the C1 cell group, only a small portion of dopamine-β-hydroxylase-positive neurons in C2, A5, and A6 and projecting to the spinal cord showed increased Fos immunoreactivity after hypotensive hemorrhage.

In a study by Krukoff *et al.* (372), approximately 33% and 16% of neurons projecting from the PVN to the NTS and the caudal ventrolateral medulla, respectively, showed Fos immunoreactivity after exposure to hypotensive hemorrhage. The targets of activated PVN neurons in the NTS and the caudal ventrolateral medulla were found to represent the same brainstem neurons that are activated by hemorrhage and that project to the PVN, suggesting that neurons in the brainstem and the PVN may reciprocally conduct cardiovascular-related information (373). About one-third of PVN neurons projecting to the brainstem were identified as nitric oxide-producing neurons, and nitric oxide is an important neurotransmitter in blood pressure regulation. In the study by Dun *et al.* (374, 375), about 60%–80% of tyrosine hydroxylase-positive and 60% of phenylethanolamine-*N*-methyltransferase-positive neurons projecting from the NTS and from the caudal and rostral ventrolateral medulla, and the rostral ventrolateral medulla to the PVN showed Fos immunoreactivity in response to hypotensive hemorrhage. In contrast, only a very few tyrosine hydroxylase- and phenylethanolamine-*N*-methyltransferase-positive neurons in the NTS displayed Fos immunoreactivity after hypotensive hemorrhage. Chan and Sawchenko (376) measured levels of mRNA for tyrosine hydroxylase in medullary catecholaminergic neurons after hypotensive hemorrhage. They reported significantly increased levels of tyrosine hydroxylase mRNA that preceded c-*fos* expression that were detected in Fos immunoreactive A1, A2, C1, and C2 cell groups. Maximal increases were detected within 0.5 to 1 and 2 h post hemorrhage in A1 and A2 noradrenergic cell groups and in C1 and C2 adrenergic cell groups, respectively. Tyrosine hydroxylase mRNA in non-Fos immunoreactive cells showed a rapid but transient increase (A1, A2, C1 cell groups) or did not change significantly over the course of the experiment (C2 cell group). As discussed by Chan and Sawchenko (354), hemorrhage-induced higher levels of tyrosine hydroxylase mRNA in noradrenergic compared with adrenergic cell groups could reflect the distinctive neuronal inputs of these brain regions. For example, more A2 than C2 neurons receive direct vagal sensory innervation, and this may explain prompt tyrosine hydroxylase mRNA responses to hemorrhage.

To separate blood pressure effects from those associated with changes in blood volume, Krukoff *et al.* (373) investigated the effects of isovolemic hypotension induced by administration of nitroprusside and hypovolemic hypotension on c-*fos* expression in the ventrolateral medulla and the NTS. No significant differences were found in patterns or numbers of activated Fos immunoreactive neurons in the NTS and the ventrolateral medulla after hypotensive hemorrhage or nitroprusside treatment. More than 90% and 75% of Fos immunoreactive neurons in the NTS and the ventrolateral medulla, respectively, did not project to the PVN. In the recent study by Chan and Sawchenko (354), withdrawal of 15% of total blood volume that caused hypotension provoked significant and progressive increases in the number and distribution of Fos immunoreactive nuclei and c-*fos* mRNA in the NTS, A1, A5, and C1 cell groups, the locus coeruleus, and the lateral parabrachial nucleus. Both Fos immunoreactivity and c-*fos* mRNA first appeared 0.5 h after the beginning of hemorrhage. Fos immunoreactivity peaked at 2.0–2.5 h and c-*fos* expression peaked at 1.0–1.5 h in the medulla and the pons. The characterization of Fos immunoreactive neurons showed that approximately 20% and 86% of Fos immunoreactive cells in the NTS and the ventrolateral medulla, respectively, displayed dopamine-β-hydroxylase immunoreactivity. In pontine regions, approximately 22% and 35% of Fos immunoreactive cells in the locus coeruleus and the A5 cell groups, respectively, were also found to be immunoreactive for dopamine-β-hydroxylase. Cells displaying both Fos and dopamine-β-hydroxylase immunoreactivity were not identified in the parabrachial nucleus. Nitroprusside-induced hypotension yielded a pattern of c-*fos* expression similar to that seen after hemorrhage, except in the A1 cell group, area postrema, and the subpostrema regions of the NTS, where the responses were muted or lacking. These results are consistent with the fact that these structures are involved in volume-induced neuroendocrine responses; A1 afferents regulate vasopressin secretion, and the area postrema is involved in regulation of the renin-angiotensin system (354). Moreover, several previous studies showed no effect of nitroprusside on plasma vasopressin levels (377–379). Different intensities and duration of hemorrhage could explain different results across these studies.

The finding that less than 50% of Fos positive PVN neurons project to the ventrolateral medulla, the NTS, or the thoracic spinal cord suggests that PVN neurons projecting to other brain regions may also be involved in activation of neuronal circuitry upon exposure to hemorrhage. Nevertheless, these results suggest that brainstem and spinally projecting neurons in the PVN or the rostral ventrolateral medulla are an important component of brain circuits involved in neuroendocrine and autonomic adjustments to hemorrhage.

In studies by Chan and Sawchenko (354) and Badoer *et al.* (380), hypotensive hemorrhage, but not nitroprusside administration, gave rise to increased Fos immunoreactivity in the area postrema and the subpostrema region. Systemic infusion of sarile, an angiotensin receptor antagonist, disrupted hemorrhage-induced c-*fos* expression in both regions, suggesting the presence of angiotensin binding sites in these regions. The anatomical connections between the area postrema and the NTS (381) and the finding that hemorrhage-induced c-*fos* expression in the NTS is attenuated by admin-

istration of sarile suggest that the area postrema participates in hemorrhage-induced neuroendocrine regulation.

Hemorrhage was also shown to induce Fos expression in several other brain regions (354, 367, 371, 375, 380). After hypotensive hemorrhage, a large number of Fos immunoreactive cells were observed in the supraoptic and dorsomedial hypothalamic nuclei, the bed nucleus of the stria terminalis, the central amygdaloid nucleus, the preoptic area, the bed nucleus of the lamina terminalis, the piriform cortex, several auditory and precerebellar nuclei, the spinal trigeminal nucleus, and the spinal intermediolateral cell column (Table 1). A few Fos-positive neurons were occasionally noted in various thalamic nuclei, tegmental nuclei, and the raphe nuclei. Double-labeling experiments showed that most of the Fos immunoreactive neurons in the supraoptic nucleus but not in the suprachiasmatic nucleus were immunopositive for vasopressin, supporting the idea that expression of Fos is stimulus-specific (375).

2. Effects of hemorrhage on the hypothalamo-pituitary-adrenocortical axis. The HPA axis is activated upon exposure to acute and chronic hemorrhage as is evident from several previous reports. CRH mRNA expression, portal plasma levels of CRH, and plasma ACTH and corticosteroids are all increased after exposure to hemorrhage (27, 304, 345–347, 356, 357, 382–385). Reduced hemorrhage-induced ACTH responses in rats with surgical or chemical lesions of the PVN, the locus coeruleus, or the NTS demonstrate that these two brain regions are essential components of the neural pathways for ACTH release after hemorrhage (358, 386, 387). Experiments with dogs demonstrate that hypotensive hemorrhage-induced increases in plasma corticosteroid levels are not always preceded by measurable increases in plasma ACTH concentrations above control, suggesting that initial (first 10–15 min) corticosteroid responses to hemorrhage may be stimulated by a factor(s) other than ACTH (356). Similarly, using *in vivo* microdialysis in the PVN, we found only small changes in extracellular concentrations of NE, a potent CRH stimulator, after exposure to hypotensive hemorrhage. Thus, we have proposed that other neurotransmitters, factors, or neuronal pathways conveying information into the PVN or bypassing it completely participate in hemorrhage-induced activation of the HPA axis. In response to osmotic stimuli, increased expression of CRH and CRH type 1 receptor have been observed in the magnocellular paraventricular and supraoptic nuclei (387a).

Experimentally induced hypotensive hemorrhage has been shown to increase vasopressin and oxytocin release in the PVN (388–390), portal plasma levels of vasopressin and oxytocin (382, 391, 392), and plasma vasopressin, oxytocin, PRL, renin, and interleukin-6 levels (356, 382, 388, 393–397). Hemorrhage-induced increases in c-*fos* expression in PVN vasopressin and oxytocin neurons have been repeatedly demonstrated (398–400). Plasma levels of vasopressin in rats with lesions of the hypothalamic supraoptic nuclei do not increase to levels attained in sham-operated rats after hypotensive hemorrhage, suggesting that the supraoptic nucleus is the primary regulatory site for vasopressin release in response to this stressor (401).

Deafferentation of the posterior hypothalamus reduces PRL responses to hemorrhage but not to immobilization stress (394). An anterolateral knife cut around the mediobasal hypothalamus eliminates both hemorrhage- and immobilization stress-induced PRL responses. In contrast, denervation of the posterior pituitary only slightly reduces PRL responses after immobilization stress but completely abolishes PRL responses to hemorrhage. These experiments support the existence of stressor-specific pathways and neuroendocrine responses (394).

Using *in vivo* microdialysis in freely moving rats, we have recently reported effects of nonhypotensive and hypotensive hemorrhage on NE release in the PVN (130). In response to both nonhypotensive and hypotensive hemorrhage, there were only modest increments in microdialysate NE levels. In contrast, several previous studies reported increased extracellular NE levels in various hypothalamic nuclei or regions after hemorrhage. In a study by Morris *et al.* (402), hypotensive hemorrhage resulted in a nonsignificant increase in extracellular NE in the rat PVN, but in the presence of desimipramine, there was a significant NE release when compared with baseline levels. Van Huysse and Bealer (403) demonstrated increased extracellular NE levels in the PVN/anterior hypothalamic area and in the dorsomedial medulla in rats after hypotensive hemorrhage and isovolemic hypotension. Thrivikraman *et al.* (404) have shown increased catecholaminergic activity in the cat PVN during hemorrhage but they did not determine which catecholamine was released. Using the push-pull cannula technique in conscious rats, Qualy and Westfall (405) demonstrated increased NE release in the PVN after administration of nitroprusside. Different experimental conditions, especially the fact that dialysate NE levels in several studies described above represented NE released from a number of brain sites/nuclei rather than from the PVN alone, most likely contributed to different results across these studies.

In the magnocellular subdivision of the PVN, hemorrhage is known to induce NE release, whose role is predominantly stimulatory to vasopressin neurons (406, 407) since treatment with 6-hydroxydopamine eliminates activation of vasopressin neurons upon stimulation of the A1 area (408). Increased NE turnover has also been described in the locus coeruleus (409); however, the functional significance of activation of this brain region is unclear since its hypothalamic and spinal projections do not appear to modulate the adaptive neuroendocrine and autonomic responses to hemorrhage (65). This may be partially explained by our recent finding that only a small portion of locus coeruleus noradrenergic neurons project to the PVN (131). Furthermore, elimination of baroreceptor afferent input to the brain produced by chronic lesions of the NTS does not alter vasopressin release during hypotensive hemorrhage in conscious rats (410).

As originally suggested by Blessing and Willoughby (411) and discussed in detail by Chan and Sawchenko (354), one possible pathway for regulation of paraventricular vasopressin upon exposure to hypovolemia involves stimulation of cardiac volume receptors. Their stimulation results in stimulation of vagal afferents that in turn stimulate A1 noradrenergic neurons, which project to the paraventricular magnocellular subdivision and to the supraoptic nucleus to facilitate release of vasopressin. This is further supported by

studies of Makino *et al.* (364) and Lightman *et al.* (363, 365), who demonstrated decreased plasma vasopressin concentrations upon exposure of rats to hemorrhage after surgical lesions of either the medial forebrain or the dorsal noradrenergic bundle. Furthermore, Head *et al.* (412) and Gieroba *et al.* (413) found marked reductions in plasma vasopressin release after electrolytic lesions or chemical blockade of the A1 area in rabbits or rats and Day and Renaud (408) reported that stimulation of the A1 area enhanced the activity of vasopressin-secreting supraoptic neurons. The activation could be prevented by local injection of 6-hydroxydopamine, although it seems that during severe hemorrhage, vasopressin cells are activated by additional vasopressin stimulatory pathways that bypass the A1 region (414, 415).

3. Proposed existence of neuronal circuits/pathways activated by hemorrhage. Hemorrhage rapidly activates the HPA axis. Both parvicellular CRH and magnocellular vasopressin neurons in the PVN are involved in this activation. In response to acute hemorrhage, increased CRH mRNA synthesis has been demonstrated in the PVN (384) and increases in CRH concentrations in the hypophyseal portal blood (416).

Signals from peripheral baro-, osmo-, and angiotensin II receptors converge to a neuronal circuit in the hypothalamus and the preoptic area. To activate the HPA axis, at least three neuropeptides, angiotensin II, atrial natriuretic polypeptide, and vasopressin, are involved. Inputs to the preoptic-hypothalamic neuronal circuit could be 1) humoral, or 2) neuronal (Fig. 13).

a. Humoral inputs. Extracellular dehydration caused by acute hemorrhage (for review, see Ref. 417) increases plasma angiotensin II concentrations and alters the activity of angiotensin II receptors in the subfornical organ and the area postrema. These two circumventricular organs lie outside the blood-brain barrier, and they express angiotensin II receptors at the highest level in the central nervous system (for review see Ref. 418). Both circumventricular organs are sensitive to circulating angiotensin II (354, 419), and angiotensin II as well as hemorrhage elicit Fos induction in their neurons (354, 420). From here hemorrhage-elicited humoral signals are transported further by neuronal routes: subfornical cells project to the preoptic-hypothalamic circuit while the area postrema neurons reach the NTS (Fig. 13).

b. Neuronal pathways. 1) Cells in the subfornical organ project to magnocellular supraoptic and paraventricular nuclei either directly or their signals are relayed by atrial natriuretic polypeptide-containing neurons in the organum vasculosum laminae terminalis and the medial (periventricular) preoptic nucleus (421). Transection of subfornical organ neuronal connections diminishes the pressor response to intravenous infused angiotensin II (422, 423). Hemorrhage activates vasopressin- and oxytocin-synthesizing hypothalamic neurons and leads to the release of these substances from the posterior pituitary. Unilateral lesions of the ventrolateral catecholaminergic system significantly reduced the hypotensive hemorrhage-induced activation of hypothalamic vasopressin, oxytocin, and medial parvocellular para-

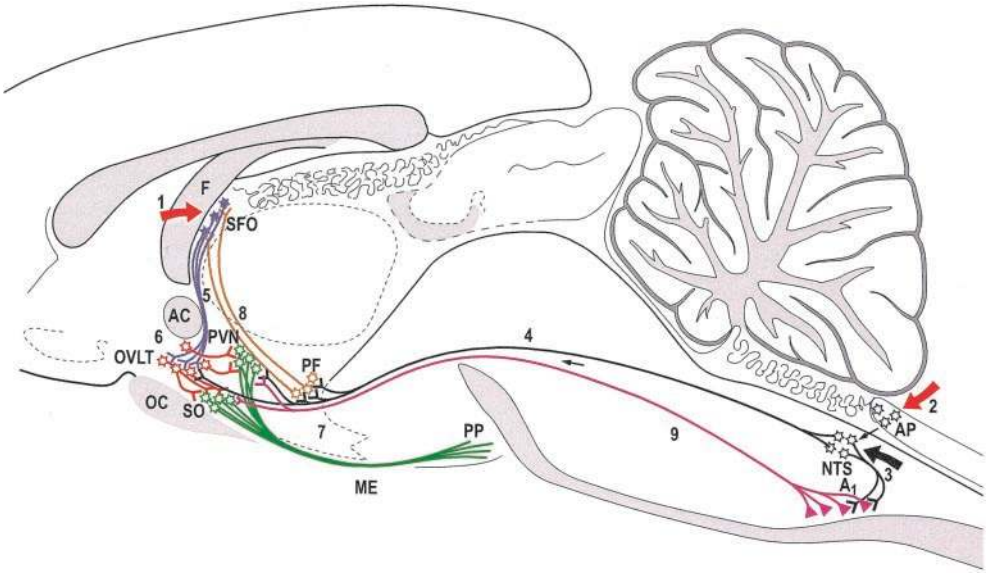

FIG. 13. Hypothalamic neuropeptidergic circuit in the organization of body fluid and mineralocorticoid homeostasis. Humoral and neuronal inputs to the hypothalamic regulatory circuit. 1, Humoral inputs to the subfornical organ; 2, humoral inputs through the area postrema; 3, viscerosensory neuronal inputs to the nucleus of the solitary tract; 4, ascending projections from the nucleus of the solitary tract to the perifornical and the paraventricular nuclei, as well as to the anteroventral third ventricle area; 5, angiotensin II-containing neurons in the subfornical organ with their projections to the medial preoptic area; 6, atrial natriuretic polypeptide-containing neurons in the organum vasculosum of the laminae terminalis and the medial preoptic area with supraoptic and paraventricular projections; 7, supraoptic and paraventricular vasopressinergic projections to the posterior pituitary; 8, angiotensin II-containing projections from the perifornical nucleus to the subfornical organ; 9, ascending noradrenergic fibers from the ventrolateral medulla to preoptic and magnocellular hypothalamic nuclei. AC, Anterior commissure; AP, area postrema; F, fornix; ME, median eminence; NTS, nucleus of the solitary tract; OC, optic chasm; OVLT, organum vasculosum of the laminae terminalis; PF, perifornical nucleus; PP, posterior pituitary lobe; SFO, subfornical organ; SO, supraoptic nucleus.

ventricular (probably CRH) cells (363–365, 413, 424). 2) In addition to circulating angiotensin II, which may reach the NTS through the area postrema, neuronal afferents from cardiac and arterial baroreceptors terminate on NTS neurons. These receptors are activated by reduced blood pressure and blood volume caused by acute hemorrhage. 3) Information from the NTS may be transmitted to the PVN through either catecholaminergic (65–67, 85) or peptidergic neurons (94, 95). 4) Aminergic neurons in the caudal ventrolateral medulla (A1 noradrenergic and C1 adrenergic cell groups) are preferentially activated by hypovolemic input (354). These neurons receive noncatecholaminergic neuronal input from the NTS (67, 197). A1 and C1 neurons contribute to the noradrenergic and adrenergic innervation of the neuroendocrine hypothalamus. The A1 cell groups constitute the major source of ascending noradrenergic projections to vasopressinergic neurons in the hypothalamus (65, 67, 408, 425). The activity of A1 neurons is negatively related to baroreceptor input (411, 426). 5) NTS neurons innervate hypothalamic perifornical cells that project to the subfornical organ (423). This route may provide neuronal feedback to angiotensin II-containing cells in the subfornical organ, or it may synchronize baroreceptor-related humoral signals to brain angiotensin II receptors (Fig. 13).

Paraventricular, perifornical, and dorsal posterior hypothalamic neurons project to sympathetic preganglionic neurons in the thoracic spinal cord (see *Section V.C.3*). Through these descending systems the sympathoadrenal system can be activated and involved in hemorrhage-induced stress responses.

E. Pain stress

Subcutaneous injection of formalin is an excellent tool to investigate pain-evoked stress responses (427). A small volume (0.015–0.2 ml/100 g body weight in rats) of 1–5% formalin injected subcutaneously into the paw elevates plasma ACTH, corticosterone, and catecholamine levels immediately and induces marked CRH expression in the PVN (4, 130, 428–431). Formalin administration induces an acute phase of nociception (up to 1–5 min) followed by a second stage, termed "tonic," which lasts for about 1 h (432). This effect is mediated by C-fiber afferents that induce excitatory responses in relevant dorsal horn neurons. Here, a non-*N*-methyl-D-aspartate ionotropic glutamate receptor (kainate GluR5 receptor subtype) mediates the nociceptive response to formalin (433).

Other painful stimuli, like mustard oil (228) or sc injection of capsaicin (19), are used less frequently to investigate pain-induced c-*fos* activation in the central nervous system.

1. c-fos Expression in the central nervous system after pain stress. Strong c-*fos* activation was observed in several brain regions 30–120 min after administration of formalin, indicating that numerous systems were influenced by this stressor (Table 1). The distribution patterns of Fos immunoreactive neurons in the brain and the spinal cord are quite similar in animals subjected to pain or immobilization stress:

a. Spinal cord. Pain induces neurons to express c-*fos* in a site-specific manner in areas of the spinal cord (Fig. 14): in the marginal layer (lamina I), the outer part of the substantia gelatinosa (lamina IIo), deep layers (laminae V and VI), lamina X, and the intermediolateral cell column (29, 63, 107, 191, 229, 430, 434–437). These regions are predominantly implicated in the reception, processing, and transmission of nociceptive information. Labeled cells extend from lumbar (L3) to sacral (S1) segments. Fos-immunopositive cell nuclei were detected only ipsilateral to the formalin injection (430).

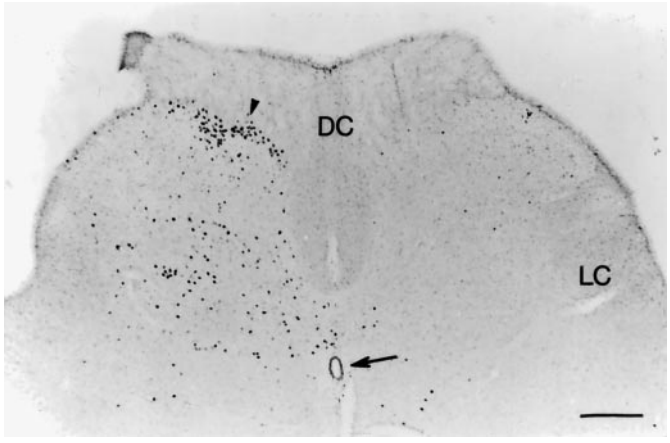

FIG. 14. Fos-immunoreactive cell nuclei in dorsal horn neurons (segment lumbar 4) 60 min after a 4% formalin injection (subcutaneous) into the hind paw. Fos-immunoreactive cells are concentrated mainly in the marginal layer (lamina I and Iio) and nociceptive neurons in laminae IV, V, VI, and X, ipsilateral to the site of the injection. DC, Dorsal columns; LF, lateral funiculus. The central canal is indicated by an *arrow*. *Bar scale*: 200 μm.

b. Central catecholaminergic and other systems. In the medulla oblongata 60 min after a unilateral formalin injection, strong bilateral labeling was seen in the ventromedial medulla (Table 1). Here, both epinephrine-containing C1 and norepinephrine-containing A1 neurons express c-*fos* in response to formalin, similar to the pattern after immobilization (19, 26, 63, 107). A fairly high number of neurons were Fos labeled in the NTS, almost all of them in the locus coeruleus and the lateral parabrachial nucleus bilaterally (26, 63, 107, 229). Formalin exerted effects on Fos activity in other pontine noradrenergic neurons (A5 and A7 cell groups), also bilaterally (26). The bilateral c-*fos* responses in brainstem and forebrain nuclei after a unilateral injection of formalin into the fore- or hindlegs clearly indicates a cross-over of pain-conducting fibers below or at the level of the medulla oblongata. This condition is supported by other observations: unilateral surgical transection at the medullary-spinal cord junction did not influence formalin-induced plasma corticosterone and ACTH levels or release of NE in both sides of the PVN (430). In hemisected rats, a formalin injection elicited bilateral c-*fos* expression in the PVN and thalamic nuclei in a pattern that was similar to sham-operated rats (430). Similar to a formalin injection, in response to footshock, Fos immunoreactivity was strongly induced in noradrenergic neurons of all brainstem catecholaminergic cell groups, as well as in central gray neurons and serotoninergic neurons in the dorsal raphe nucleus (438).

Formalin also activated neurons in the periaqueductal central gray (107). Endogenous opioid neurons of this region are

implicated in pain modulation (96, 439, 440). These neurons receive nociceptive inputs from the spinal cord through the spinomesencephalic tract (441, 442) and exert their antinociceptive action through rostral ventromedial medullary neurons from where facilitatory signals descend to the spinal serotoninergic system (443). These medial ventromedial medullary neurons are activated (through GABAergic disinhibition) by enkephalin and dynorphin neurons of the periaqueductal central gray. Some of the neurons in the reticular formation and contribute to persistent pain by enhanced release of substance P from primary afferent nociceptive fibers in the spinal cord (444).

After a formalin injection in the midline and intralaminar thalamic nuclei, marked expression of c-*fos* is induced (Fig. 15) where the spinoreticulothalamic fibers terminate (26, 28, 63, 229). This area includes the central medial, thalamic paraventricular, rhomboid, anterodorsal, mediodorsal, intermediodorsal, and reuniens thalamic nuclei and the medial subdivision of the lateral habenular nucleus. These sensory-discriminative pain fibers carried by the spinothalamic and trigeminothalamic tracts terminate in the ventral posterolateral and posteromedial thalamic nuclei. These nuclei showed c-*fos* activation on the contralateral side after a formalin injection (105).

Painful stimuli resulted in marked c-*fos* expression in the hypothalamus, particularly in the medial parvicellular subdivision of the PVN (Fig. 8B), overlapping an area where CRH-immunoreactive neurons are highly concentrated (26, 63, 107, 229). Neurons in the lateral hypothalamus, mainly in the middle part of the hypothalamus and in the medial preoptic and the supramamillary nuclei, were also activated by formalin injections similar to previous reports (107).

The amygdala has been considered to play a role in stress-related changes in pain sensitivity. It may exert an antinociceptive action through the central gray-medial ventromedullary system (445). Among the amygdaloid nuclei, formalin elicited c-*fos* expression in the medial and central amygdaloid nuclei. Like formalin-evoked pain, foot shock also induced c-*fos* expression in the amygdala (106).

Noxious stimulation of the cornea in rats produced a characteristic distribution of Fos in the spinal trigeminal nucleus (446).

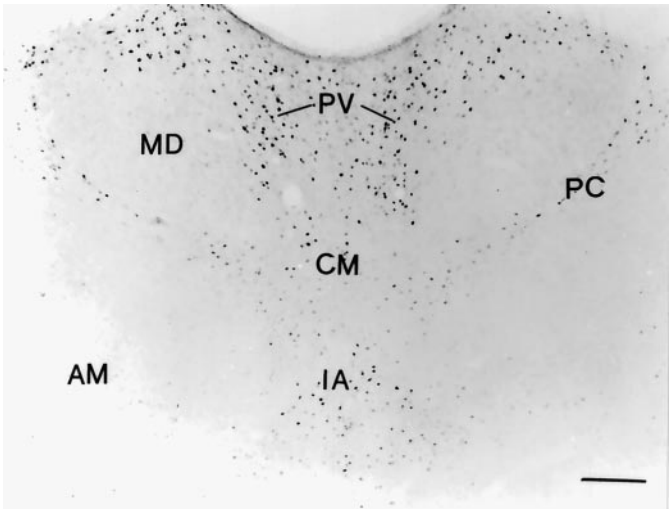

FIG. 15. Fos-immunoreactive cell nuclei of midline and intralaminar thalamic nuclei 60 min after a 4% formalin injection (sc) into the hind paw. A - coronal section through the anterior (1.8 mm caudal to the level of the bregma) part of the dorsal thalamus. AM, Anteromedial thalamic nucleus; CM, centromedian thalamic nucleus; IA, interanteromedian thalamic nucleus; MD, mediodorsal thalamic nucleus; PC, paracentral thalamic nucleus; PV, paraventricular thalamic nucleus. *Bar scale*, 200 μm.

2. In vivo microdialysis studies during painful stimuli. Using an *in vivo* microdialysis technique, we also examined the effects of 4% formalin administration into the hind paw on NE release in the PVN. Subcutaneous injection of 4% formalin caused marked increases in extracellular PVN NE, dihydroxyphenylglycol, and dihydroxyphenylacetic acid concentrations (4, 130). Formalin-induced increases in PVN NE concentrations reached a peak 30 min after injection. Spinomedullary hemisection did not affect baseline and formalin-induced increases in PVN NE concentrations (430). Pontomedullary hemisections did not affect formalin-induced immediate and marked NE release in the PVN contralateral to the knife cut (430). Similar to intact rats, 5- to 8-fold increases in NE levels were measured independently of whether formalin was injected into the right or the left leg. The hemisection attenuated formalin-induced elevations of NE in the PVN ipsilateral to the knife cut, regardless of whether formalin was injected into the left or the right leg. In these animals, PVN NE concentrations were twice as low as those measured in the contralateral PVN, but twice as high as in rats with subcutaneous saline injection. Our data further indicate that medullary NE afferents that project to the PVN ascend mainly on the ipsilateral side. As indicated by Fos expression studies, the main portion of formalin-induced NE release from the PVN is derived from the A1 catecholaminergic cell group and a smaller portion is derived from catecholaminergic inputs from the locus coeruleus. By simultaneous measurements of plasma ACTH concentrations after formalin injection, we demonstrated a positive correlation between NE release in the PVN and plasma ACTH responses (130), suggesting that PVN NE is stimulatory to PVN CRH neurons during formalin-induced pain.

3. Proposed existence of neuronal circuits/pathways activated by pain. There appear to be numerous neuronal pathways leading to the hypothalamus, mainly to the PVN and the median eminence, which convey pain-related signals. The nociceptive signals may reach the hypothalamo-pituitary system directly through the spinohypothalamic tract, or through activation of brainstem catecholaminergic and noncatecholaminergic neurons (Fig. 16).

First-order sensory neurons (neuronal perikarya in dorsal root and Gasserian ganglia) terminate in various cell types (directly on projection neurons or excitatory interneurons) in the dorsal horn of the spinal cord and the sensory trigeminal nucleus. Second (and/or third) order nociceptive fibers run in the spinothalamic, spinoreticulothalamic, spinoreticular, and spinohypothalamic tracts. The spinothalamic tract, which is a phylogenetically younger structure and well developed in humans, projects from the spinal cord directly to the ventral posterolateral thalamic nucleus, from where it projects to sensory-discriminative cortical areas. The spino-

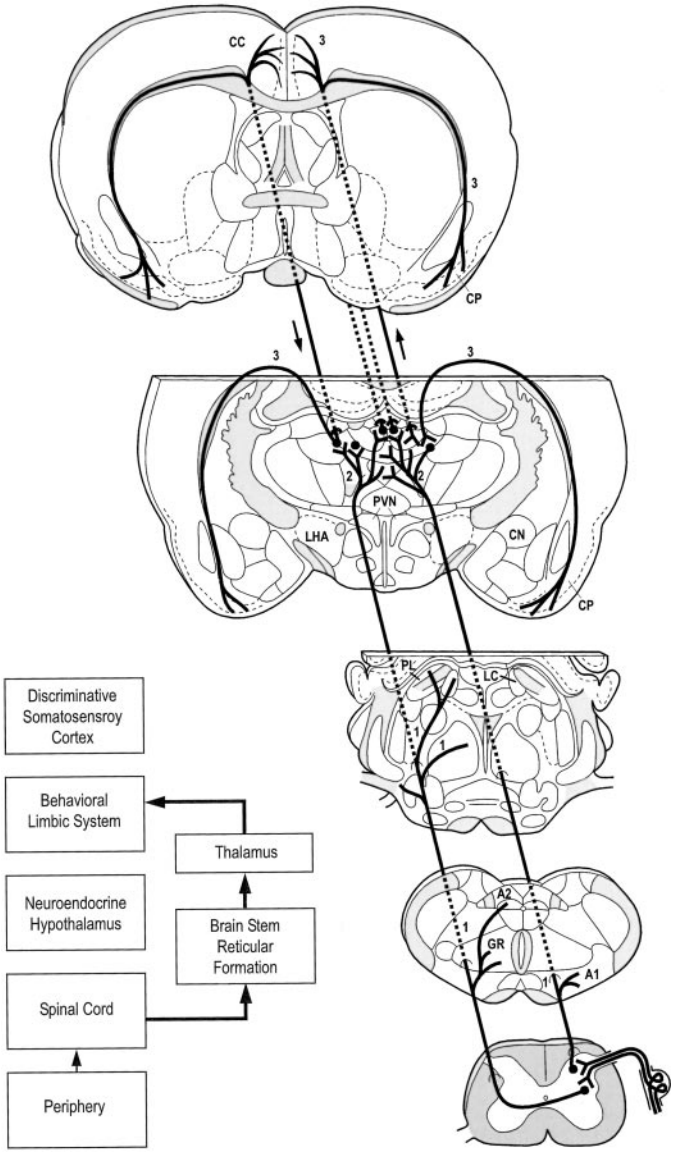

FIG. 16. Spinoreticulothalamic tract. Ascending nociceptive pathways to the hypothalamus and the limbic system. 1, Projections to brainstem catecholaminergic and reticular formation neurons; 2, nerve terminals in the intralaminar and midline thalamic nuclei; 3, thalamic projections to the cingulate and piriform cortex. A1, A2, A1 and A2 noradrenergic cell groups; CC, cingulate cortex; CN, central amygdaloid nucleus; CP, piriform cortex; GR, gigantocellular reticular nucleus; LC, locus coeruleus, LHA, lateral hypothalamic area; PL, lateral parabrachial nucleus.

reticulothalamic tract is an older structure (it is also called the paleospinothalamic tract or medial spinothalamic tract), a multisynaptic system, which terminates in several neurons in the reticular formation and finally reaches the midline and intralaminar thalamic nuclei. The final destination of these projections is the motivational effective (limbic) cortical areas (cingulate, piriform, and entorhinal cortex). The spinohypothalamic pathway (183, 185) conducts direct signals up to the lateral hypothalamus from where they are relayed to the PVN.

Visceral pain signals are mainly carried by vagal and glossopharyngeal nerves to the NTS. From here, this information is relayed bilaterally to the parabrachial nucleus and further up to the thalamus. The pathway terminates in the viscerosensory cortical areas (prelimbic, intralimbic, and anterior cingulate cortex).

Directly or indirectly, pain-related ascending signals reach the PVN, and they are crucial to the organization of stress responses. Complete unilateral ponto-medullary brainstem surgical cuts reduced CRH immunoreactivity (64, 131, 177, 178, 447–449) and the expression of CRH mRNA in the PVN (148, 182).

Some nociceptive signals are relayed via brainstem catecholaminergic neurons and are conveyed by the ascending ventral noradrenergic bundle to the hypothalamus. Lesions of this bundle have a blocking effect on responses to stressful, including noxious, stimuli (62). The majority of noradrenergic fibers ascend on the same side and innervate the ipsilateral PVN (64). Catecholaminergic input to the PVN is known to have a significant effect on stress-induced CRH expression and release (24).

Spinal projections from nociceptive neurons to the ventrolateral medulla, including the A1 noradrenergic and C1 adrenergic cell groups, have been demonstrated with tract-tracing techniques (187, 188, 190, 191). All segments of the spinal cord contribute to the projection to the ventrolateral medulla. The ventrolateral medullary neurons (including A1 and C1 cells) are involved in central processing of interoceptive information. They receive visceral sensory input gated initially through the NTS (195).

Retrograde transport studies have also shown that a considerable number of spinal cord neurons, including nociceptive neurons in the superficial layers of the dorsal horn, project to the NTS (93). In addition to these, the NTS, which includes the A2 noradrenergic and C2 adrenergic cell groups, also receives vagal and glossopharyngeal viscerosensory inputs (450). NTS neurons project to the ventrolateral medulla (65, 67, 77, 93, 197), to the locus coeruleus (67, 451), and to diverse forebrain regions including the PVN (65, 67, 77, 95, 197). Signals may reach the hypothalamus and the amygdala directly or through the parabrachial nuclei (97). Several non-catecholaminergic neurons in the NTS receive nociceptive signals from spinal cord neurons, as has been demonstrated by formalin-induced c-*fos* expression in tyrosine hydroxylase-negative NTS cells (29, 452). Axons primarily from peptidergic neurons terminate in the forebrain, including the PVN (90, 94, 95).

Nociceptive fibers (or their axon collaterals) may reach the locus coeruleus through the spinoreticulothalamic tract. Locus coeruleus neurons increase their activity in response to painful stimuli (70). As a sign of the activation, marked c-*fos* expression has been demonstrated in locus coeruleus neurons after formalin injection (26, 107, 453), or after other painful stimuli such as footshock (438). Tyrosine hydroxylase-positive cells in the locus coeruleus project to the PVN (67, 454, 455). In addition, CRH may act as a neurotransmitter to activate noradrenergic neurons in the locus coeruleus (456–458). CRH content in the locus coeruleus is increased after acute or chronic pain stress (456).

The parabrachial nuclei appear to function as a secondary integration site for autonomic regulation (cardiovascular,

respiratory, gustatory) and also for nociception. Fibers from the superficial laminae of the lumbar dorsal horn and from the spinal trigeminal nucleus ascend to the parabrachial nuclei along with the ventral spinocerebellar tract (98). The parabrachial nuclei are also the main sites for the relay of visceral information from the NTS to the forebrain.

Primary afferent fibers arising through the trigeminal, facial, glossopharyngeal, and vagal fibers are collected in the spinal trigeminal tract and terminate along the rostro-caudal axis of the spinal trigeminal nucleus. From here, secondary sensory neurons join the spinothalamic tract (lateral trigeminothalamic tract) and pass through the lateral hypothalamus on their way to the thalamic nuclei. Reportedly, nociceptive signals carried by the trigeminal nerve and the tract may reach the lateral hypothalamus (459).

The physiological response to painful stimuli is conducted by two major systems (Fig. 16): 1) activation of the HPA axis, and 2) activation of the central autonomic system. The role of PVN neurons is significant in both pathways. Here, CRH is one of the most potent neuropeptides in the stress response. It acts as a neurohormone in the HPA axis and mediates transmission through neuronal pathways that regulate autonomic outflow and visceral activity. The topography of the fibers in the formalin-evoked stress response is similar to the pattern that has been described for immobilization stress.

VI. Stressor-Specific Activation of Other Neuroendocrine Systems

Many other neuroendocrine axes are also activated upon exposure to acute or chronic stressors (7, 53, 460, 461). In terms of the hypothalamic-gonadal axis, exposure of animals and humans to acute stress is associated with a small and often short-lived increase in plasma LH and androgens (462, 463). Acute stress-induced increases in androgen appear to be caused by an alteration in plasma volume and decrease in androgen metabolic clearance, rather than as a result of a true alteration of androgen release (462). The mechanisms by which LH is increased under various stress conditions are not clear, but one possibility is that high levels of ACTH may stimulate GnRH neurons (462). Stress-induced responses are also dependent on estrogen levels; thus, animals in proestrus or gonadectomized animals do not show LH rise upon exposure to acute stress, but instead a quick decrease can be found (460). Biogenic amines such as NE and serotonin and IL-1α also participate in the regulation of the hypothalamic-pituitary-gonadal axis during acute stress reaction and most likely exert inhibitory effect on GnRH neurons or LH (462). FSH may follow the same stress response pattern as LH, but often the changes are very small or do not even exist. In contrast, upon exposure to chronic stress, reproductive functions decrease. Such an inhibition occurs at all levels including the hypothalamus (inhibition of GnRH secretion), the anterior pituitary (inhibition of LH release), and gonads (sex hormones secretion). Stress-induced activation of the hypothalamic-pituitary-adrenocortical axis is one of the main factors contributing to this inhibition since CRH and glucocorticoids are known to inhibit the GnRH, and glucocorticoids themselves exert inhibitory effects at the level of anterior pituitary and gonads (462).

It is also well documented that acute exposure to various stressors (*e.g.*, hypoglycemia, exercise, pain) increases secretion of GH from the pituitary gland. In contrast, other stressors such as cold, handling, hypertonic glucose, and electric shock produce marked decrease in plasma GH levels (464). Acute glucocorticoid administration also increases GH concentrations in plasma (6). Thus, it appears that acute stress responses that are associated with acute plasma glucocorticoid elevation may increase plasma GH levels through stimulation of the GH gene through glucocorticoid-responsive elements in its promoter region (464). In contrast, chronic stress inhibits GH release from the anterior pituitary gland and increased resistance of peripheral tissues to insulin-like growth factors (*e.g.*, IGF-I). Such inhibition may occur via CRH-induced increase in somatostatin secretion. Thus, dwarfism that occurs in the setting of severe chronic psychosocial deprivation may reflect chronically inhibited growth axis (for review, see Ref. 6). How catecholamines and other neurotransmitters contribute to stress-induced activation or inhibition of GH secretion is unclear.

In terms of the hypothalamic-pituitary-thyroid axis, acute (most often) stressors with high intensity induce inhibition of the thyroid axis. This is reflected by the decrease in release/production of hypothalamic TRH, decreased production/secretion of thyroid-stimulating hormone, and by inhibition of conversion of T_4 to more biological active T_3 in peripheral tissues (for review, see Ref. 6). CRH, somatostatin, and cytokines (*e.g.*, IL-1 and IL-6) most likely contribute to acute stress-induced inhibition of the thyroid axis. The exception is the exposure to acute cold stress, forced swimming, and noise stress when profound thyroid axis activation occurs (12). Mechanisms by which cold-induced activation of TRH occurs are not fully understood, although catecholamines are thought to participate in this interaction.

PRL can be viewed as another anterior pituitary hormone that is increased during exposure to various stressors such as exercise, hypoglycemia, hemorrhage, immobilization, and pain stress (6, 7, 460, 461). The mechanisms by which stress increased PRL secretion remain unknown. The physiological consequences of PRL hypersecretion during stress are not clear, although it is considered that PRL may enhance immune function.

VII. Clinical Relevance of Stressor Specificity and Future Perspectives

At the present time, it is well recognized that stress plays an important role in the pathogenesis of chronic diseases. Recent studies by Chrousos and others (6) suggest that chronic diseases such as affective disorders, panic disorders, anxiety, anorexia, chronic inflammatory disorders, and gastrointestinal disturbances, among others, reflect dysregulation of stress responses and are expressed either as hyperfunctional or as hypofunctional states. In melancholic depression, hyperarousal, anxiety, hypervigilance, and insomnia reflect maladaptive generalized stress responses, including tachyphylaxis of the mesocorticolimbic system and

hypersecretion of CRH in brain (6). Females exposed to chronic stress may present with delayed puberty, anovulatory cycles, and spontaneous abortions, and their infants have increased mortality (465). In males, chronic stress induces inhibition of testosterone secretion associated with abnormal spermatogenesis and decreased libido (466). Psychosocial dwarfism is another example of the effects of chronic emotional stress (467). Chronic fatigue syndrome, increased vulnerability to infection, and tumor development are examples of chronic stress-induced immunosuppression (6, 7, 468). Finally, osteoporosis and syndrome X may result from chronic hyperactivity of stress effector systems as seen, for example, in melancholic depression (6, 469). Disturbances of central noradrenergic neurons repeatedly described in patients with typical depression may play an important role in the pathogenesis of this mood disorder (469). Based on previous reports showing stimulatory role of NE on CRH neurons, it was hypothesized that stress-induced NE release in the brain increases CRH neuron activity, which in turn feeds back to increase locus coeruleus firing (470). CRH acts either directly via its CRH receptors or by stimulating the HPA axis (for review, see Ref. 470). Recent studies show that transgenic mice overexpressing CRH exhibit an increase in anxiogenic behavior (471). In contrast, administration of a CRH receptor antagonist reduces anxiety behavior, and these drugs are now potential targets for a new generation of antidepressants (472). In terms of direct glucocorticoid action on brain function, transgenic mice in which glucocorticoid receptor gene expression is decreased serve as an excellent model to study the role of glucocorticoids in behavioral changes that resemble signs and symptoms found in human depression (473, 474). These behavioral deficits can be reversed by administration of antidepressants that inhibit monoamine oxidase (473). This inhibition results in increased synaptic cleft NE levels and glucocorticoid receptor signaling efficiency that may not necessarily depend on higher NE concentrations (for review, see Ref. 470).

To understand fully how each stressor operates to elicit particular organ dysfunction, one must determine whether stressor-specific pathways/circuits exist and whether stress-induced activation or inhibition of such pathways is associated with abnormal organ function, known as stress disorders. Our present studies are a starting point in developing a foundation of research in this field and for introducing for the future specific therapies for stress-related disorders.

Differences in neuroendocrine responses of an organism upon exposure to various stressors are not consistent with the existence of a unitary stress syndrome. Instead, we have introduced a new proposal that each stressor has its own unique "signature." Thus, stress may be viewed as a response that reflects the existence of specific central anatomical and functional circuits. This view of stressor-specific circuits is, however, not new and has been discussed recently by several researchers working in the field of stress research (21, 53, 469, 475–482). For example, Herman and Cullinan (21) outlined interactions between stress-sensitive brain circuitry and neuroendocrine neurons of the PVN, especially as they relate to activation of the HPA axis. They divided stressors into two categories: those involving an immediate physiological threat, called systemic stressors, and those requiring interpretation by higher brain structures, called processive stressors. According to their theory, systemic stressors are relayed directly, probably via brainstem catecholaminergic projections, to the PVN. In contrast, processive stressors are channeled through limbic forebrain sites that upon their activation or inhibition affect the PVN via GABA-containing neurons. Recently, Li *et al.* (479) suggested the existence of different central circuits upon exposure to systemic *vs.* emotional stressors. In the biochemical switching hypothesis, Swanson (478) proposed that upon exposure to a stressor, stressor-specific changes in synthesis, transport, and storage of individual neurotransmitters exist, which determine a stressor-specific ratio of neuroactive substances at the axon terminal and serve as the final signal given to an effector system.

In our studies, we also demonstrated stressor-specific responses of the HPA axis and the sympathoadrenal system. In contrast to other studies, our experiments combined *in vivo* and *ex vivo* measurements of central and peripheral endocrine responses to various stressors. Figure 17 presents our proposed mechanisms by which noradrenergic neurons in the PVN may be involved in the regulation of the HPA axis after exposure to different stressors. When animals are exposed to metabolic stressors (*e.g.*, hypoglycemia), activation of the HPA axis does not require noradrenergic neurons

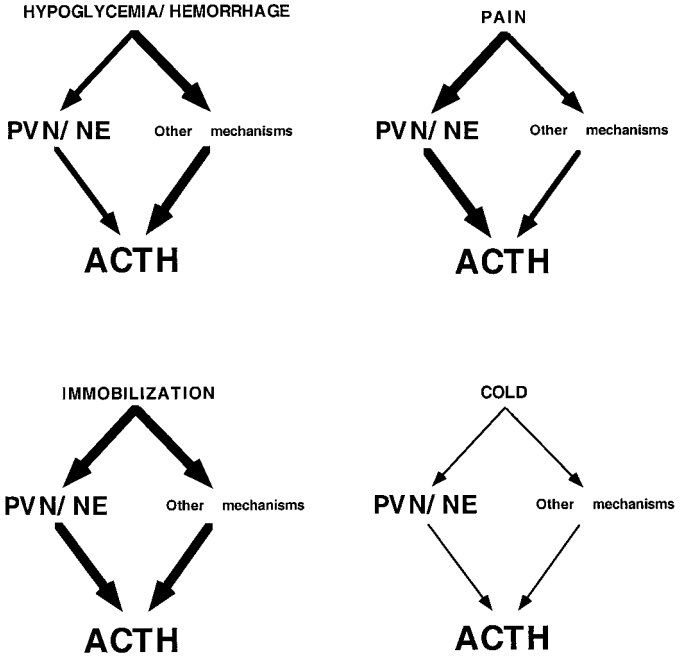

FIG. 17. Proposed schematic diagram of the role of PVN, NE, and other mechanisms on the regulation of the HPA axis during stress. Upon exposure to metabolic stressors (hypoglycemia, hemorrhage) PVN NE is only slightly elevated, and it plays a minor role in the activation of the HPA axis. Upon exposure to pain, PVN NE is increased significantly and is considered to be a potent stimulator of PVN CRH neurons. Although immobilization stress leads to a significant increase in PVN NE, a correlation between PVN NE and plasma ACTH levels suggests that immobilization stress-induced activation of the HPA axis depends on both PVN noradrenergic and PVN or extra-PVN nonnoradrenergic mechanisms. Finally, maintaining homeostasis in an organism exposed to cold stress does not require significant activation of PVN NE and the HPA axis, but the activation of other systems such as the hypothalamo-pituitary-thyroid axis is essential.

terminating in the PVN. Upon exposure to painful stressors (*e.g.*, subcutaneous formalin), NE released in the PVN is the main stimulus of CRH neurons in the PVN. Activation of the HPA axis during immobilization stress, combining physical and psychological stressors, depends on both PVN noradrenergic and PVN or extra-PVN nonnoradrenergic mechanisms. Finally, maintaining homeostasis in an organism during cold stress does not require significant activation of the HPA axis and central noradrenergic neurons but does involve activation of other systems such as the hypothalamopituitary-thyroid axis. All of these data suggest the existence of stressor-specific central pathways that participate differentially in the regulation of sympathoneuronal and adrenomedullary outflow as well as activity of the HPA axis. Our conclusions further support our view and suggestions by others (483) that the neural representations of stress and other disease-related symptoms and signs cannot be described as a matter of altered function of one brain region or neurotransmitter. Rather, it reflects the activation of several circuits to orchestrate an optimal pattern of neuroendocrine and other responses. It is very likely that one of these circuits is the primary circuit, others are subordinate, and all combine to form the functional circuit to guarantee maximal plasticity of stress responses during acute as well as chronic stress conditions. Stressor-specific formation of new connections and disconnections of subordinate circuits with the primary one, anatomical and biochemical switches within the functional circuit, previous experience of an organism to an individual stressor, and genetically programmed neuronal and cellular functions precisely tune responses to stress and maximally protect from its deleterious effects. For example, in the response to an acute stressor, the failure of the primary circuit to function properly may have disastrous effects for an organism as is evident from neuroendocrine responses in patients with hypothalamic lesions; failure of subordinate circuits may contribute to milder symptoms and signs.

We are now left with the question of how the existence of stressor-specific circuits and the involvement of individual brain regions coordinating neuroendocrine responses of stress effector systems may help us in developing novel approaches to the pathogenesis of stress-related disorders and their possible treatment. For example, as proposed by Maier and Watkins (475), upon exposure of an organism to infection, IL-1 is released from macrophages and binds to receptors on paraganglia that surround vagal nerve fibers. Specific vagal nerve fibers carry a neural signal to the NTS and from there to specific brain areas such as the hypothalamus and hippocampus. Increased local production and release of PGs in the preoptic-anterior hypothalamic area alters the activity of temperature-sensitive neurons and fever occurs. If vagotomy is performed, PG release in the preoptic region is prevented and no fever occurs (484).

Another promising area of research in which the mapping of stressor-specific pathways may markedly contribute to the future development of therapies for stress-related disorders is the development of drugs that will specifically target neurons and their products, other neurocompounds, or neuroreceptors in individual brain regions. This is well documented by recent progress in the development of novel analgesic drugs (485). After peripheral nerve injury, a variety of pain syndromes, such as causalgia, thermal hyperalgesia, or reflex sympathetic dystrophy, occurs. Nociceptive signals are carried to the dorsal horn of the spinal cord where the processing of nociceptive information, including the participation of different neurotransmitters and other substances, has been recognized and described in great detail (485). Thus, one rational strategy for altering pain processing at the spinal level would be to affect specific substances (glutamate, substance P, galanin, vasoactive intestinal polypeptide, calcitonin gene-related peptide) released from primary afferents to prevent postsynaptic spinal excitation. Recent attention has been paid to glutamate and neurokinin receptor antagonists that have been shown to have potent antinociceptive effects (485–487). Pain-induced excitation of spinal postsynaptic neurons is coupled with an increase in intracellular Ca^{2+} followed by activation of nitric oxide synthase and cyclooxygenase. Not surprisingly, spinal delivery of a cyclooxygenase-2 inhibitor or L-type Ca^{2+} channel blockers was shown to attenuate the hyperalgesic state induced by tissue injury (488, 489). Targeted drugs and toxins are another promising area of pharmacological research that may be used in the regulation of stress responses by selective inactivation of transmitter release mechanisms of the targeted cell (485). Spinal delivery of substance P-saporin, which kills local neurons expressing the NK1 receptor and, thus, produces a potent antinociception, is one example (490).

Within the context of stressor specificity, individual stressors in turn down-regulate most "housekeeping" genes while, at the same time, inducing a small set of stressor-specific genes (9). Such stress genes can produce specific proteins (*e.g.*, heat-shock proteins) that may play a critical role in preventing cells from sustaining damage and possibly undergoing apoptosis. For example, as discussed by Macario and Macario (9), cells can be stressed after exposure to heat shock. If they survive, they can tolerate a second heat shock at a much higher temperature that would otherwise be lethal for normal cells. The acquired resistance to a lethal temperature is caused by stress proteins, in this particular case by heat-shock proteins. These cells are also resistant to other stressors as well. This process is referred to as "cross-tolerance." The potential clinical applications of this phenomenon can be tremendous. One can design a specific compound that could induce a mild, innocuous stress response in various cells that would then become preconditioned cells resistant to harmful stressors. A similar analogy is seen in the process of immunization (491, 492). Thus, we predict that in the near future we may identify a hereditary or acquired deficiency in the stress response caused by "defective" stress proteins.

Introducing knockout technology has tremendous potential in medical research, since knockout animal models help describe the pathogenesis of stress-related diseases and serve as important tools to develop new therapeutic approaches. At the present time there is no knockout mouse model that is characterized by increased susceptibility to stress-related disorders. Nevertheless, CRH, or CRH receptor knockout, knockdown, or overexpression mice represent a first attempt at addressing this hypothesis (491–494). We also envision that tissue-specific knockout technology will be applied to brain research and allow us to study the involvement of specific neurocompounds in particular brain regions in stress

responses. The use of oligonucleotide antisense for specific mRNAs to prevent the expression of specific products (proteins) as a result of stressor-induced activation of individual neurons represents another approach for controlling stress responses (485).

Finally, new trends in brain imaging may help to identify stressor-specific neuroanatomical and functional circuitry. The field of nuclear medicine has evolved rapidly in recent years, especially given that new radiocompounds have been introduced to visualize a wide variety of disease processes at the cellular as well as molecular levels (495). Using positron emission tomography and functional magnetic resonance imaging in human stress research will open new directions and perspectives regarding ways to correlate stress-induced dysfunctions of peripheral organs with changes in activity of specific neurons in individual brain regions. The possibility of imaging stress-induced activity in various brain areas simultaneously and precisely will contribute further to the identification of stressor-specific circuits.

Despite the many difficulties in studying and defining stress, stress is an important area of medicine. We do not think that one magic stress hormone exists and we do not think that stress disorders can be treated simply. Nevertheless, the basic studies described in the present review illustrate the importance of continuing neuroscience research and applying new information to clinical practice. The proposed existence of stressor-specific pathways and circuits is definitely a step forward in the study of the pathogenesis of stress-related disorders. The identification of stressor-specific circuits helps us find "crucial" neurotransmitters or other neurocompounds and determine disordered sites (*e.g.*, nuclei) located within individual stressor-specific brain regions. Specific targeting of these nuclei, their genetic equipment using tissue/region-specific knockout technology or their neurotransmitters and neurocompounds will bring novel information of their involvement in the pathogenesis of stress-related diseases and may promote the development of new therapeutic approaches. Furthermore, introducing new imaging techniques such as positron emission tomography or functional magnetic resonance imaging into stress research may bring unique information about the function of the "living" human brain. New imaging techniques will also allow for simultaneous observations of stressor-specific changes in central processes such as blood flow, neurotransmitter release, and receptor binding characteristics in individual regions with responses of stress effector systems. However, this may take time, since current basic and clinical research has just begun to unravel pathophysiological mechanisms for some major stress-related diseases. Meanwhile, we hope that researchers will elaborate on this theory of stressor specificity into their research and in further explorations of the pathogenesis of stress-related diseases.

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