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Corticotropin Releasing Hormone and Imaging, Rethinking the Stress Axis

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Abstract

The stress system provides integration of both neurochemical and somatic physiologic functions within organisms as an adaptive mechanism to changing environmental conditions throughout evolution. In mammals and primates the complexity and sophistication of these systems has surpassed other species in triaging neurochemical and physiologic signaling to maximize chances of survival. Corticotropin releasing hormone (CRH) and its related peptides and receptors have been identified over the last three decades and are fundamental molecular initiators of the stress response. They are crucial in the top down regulatory cascade over a myriad of neurochemical, neuroendocrine and sympathetic nervous system events. From neuroscience, we've seen that stress activation impacts behavior, endocrine and somatic physiology and influences neurochemical events that one can capture in real time with current imaging technologies. To delineate these effects one can demonstrate how the CRH neuronal networks infiltrate critical cognitive, emotive and autonomic regions of the central nervous system (CNS) with somatic effects. Abundant preclinical and clinical studies show inter-regulatory actions of CRH with multiple neurotransmitters/peptides. Stress, both acute and chronic has epigenetic effects which magnify genetic susceptibilities to alter neurochemistry; stress system activation can add critical variables in design and interpretation of basic and clinical neuroscience and related research. This review will attempt to provide an overview of the spectrum of known functions and speculative actions of CRH and stress responses in light of imaging technology and its interpretation. Metabolic and neuroreceptor positron emission/single photon tomography (PET/SPECT), functional magnetic resonance imaging (fMRI), anatomic MRI, diffusion tensor imaging (DTI), proton magnetic resonance spectroscopy (pMRS) are technologies that can delineate basic mechanisms of neurophysiology and pharmacology. Stress modulates the myriad of neurochemical and networks within and controlled through the central and peripheral nervous system and the effects of stress activation on imaging will be highlighted.

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Introduction

Relevant Biology and Physiology

The stress system has ancient molecular roots. A critical stress mediator is Corticotropin Releasing Hormone (CRH) and it is a member of a family of stress-related peptides. It was first isolated and discovered from over 500,000 sheep hypothalami by Vale W et al. in 1981[1]. Subsequently, a series of CRH-related peptides and receptors were discovered in vertebrates and invertebrates. These peptides include urocortin 1, urocortin 2 or stresscopin-related peptide, sauvagine and urocortin 3 or stresscopin and a series of associated receptors. Vertebrates show wide expression of these molecules in the central and peripheral nervous system where they activate physiologic responses to changing environmental conditions. CRH and these peptides are integral to reproduction, migratory behavior, timing of metamorphosis and other critical, adaptations to environmental changes [2, 3, 4]. CRH and receptors are expressed in a variety of other tissues including but not limited to the immune system (cellular elements, lymphatic tissues), integument, the blood brain barrier (BBB), gastrointestinal tract and parasympathetic ganglions. The actions of CRH are principally paracrine in loco-regional tissues with indirect effects on the CNS which are beyond the scope of the review.

Increasing complexity of the central and peripheral nervous systems seen in mammals resulted in higher levels of expression of CRH, urocortins and associated receptors, as well as their expression, in immune, reproductive, endocrine and other tissues/organs gaining importance in the central integration of these systems.

An acute stress response involves an abrupt usually self-limited neurohormonal activation as a cascade effect often in response to real or perceived physical or emotional danger i.e. fight, flight or freeze. The inability to mount an adequate stress cascade holds survival disadvantages. A chronic stress state involves prolonged pathologic neurohormonal activity usually over months to years. Involvement engages C, hypothalamic pituitary adrenal axis (HPA) and SNS (and other) activity that would be out of context for the environmental conditions. Chronic stress causes desensitization of normal feedback systems; this can result in elevation of C, down-regulation of adrenergic receptors (epinephrine, norepinephrine), loss of normal circadian rhythmicity. Long term effects include immune, metabolic, cardiovascular diseases and increased susceptibility to other CNS disorders. The evolutionary benefit from the stress system involves a balance of adequate acute responses that optimize survival advantage while assuring that chronic activation and related adverse consequences is aborted.

Functional imaging technologies involving the central nervous system continue to expand and refine. Imaging is widely used to study disorders associated with or secondary to acute and chronic stress system dysfunction. Metabolic and neuroreceptor positron emission/ single photon tomography (PET/SPECT), functional magnetic resonance imaging (fMRI), anatomic MRI, diffusion tensor imaging (DTI), proton magnetic resonance spectroscopy (pMRS) are all technologies in which stress activation may impact signal sensitivity and specificity and may alter results and study interpretation. Here we hope to demonstrate areas

of imaging where stress manifests alterations in neurochemistry, cognition and anatomy and provides new considerations for understanding underlying its variability.

Physiology Interface with Stress Activation

In the CNS, CRH and CRHR1 are highly concentrated in the hypothalamus, areas of the neocortex principally the prefrontal cortex (PFC), discrete brain stem nuclei, and the extended amygdala (EA).

a) Hypothalamus

The hypothalamus regulates stress hormone release from the pituitary and control of the sympathetic nervous system (SNS) through the brainstem structures, the pontomedullary and raphé nuclei and the locus coeruleus (LC). These areas express very high levels of immune-reactive CRH (irCRH) and CRHR1 [5]. Hypothalamic CRH is released from the paraventricular nucleus through the infundibular stalk to the anterior pituitary activating CRHR1 and (with severe stress) arginine vasopressin (AVP) stimulating adrenocorticotrophin (ACTH) release. ACTH is a potent secretagogue releasing C and androgens from the adrenal cortex. There is a potent negative feedback system for the regulation of C at the levels of the pituitary, hypothalamus and the EA. Activation of the HPA with a surge of C secretion will trigger glucocorticoid receptors (GCr) in the pituitary/hypothalamus to deactivate CRH secretion and dampen the neurohormonal stress response (Figure 1). Negative feedback responsivity will fail during conditions of persistent, frequent stressors either exogenous, endogenous or both. GCr are expressed in other brain regions where they have significant effects on neurochemistry, genetic/epigenetic events and neural growth. The hypothalamus activates SNS and adrenal medullary catecholamines, epinephrine and norepinephrine. Direct autonomic innervation and circulating catecholamines stimulate the heart, vascular structures, visceral and lymphoid tissues and increase cardiovascular, metabolic, immune, and other physiologic responses to stressful stimuli [6, 7].

b) Extended Amygdala

CRH and CRHR1 are also expressed in the extended amygdala (EA); it is a complex structure composed of discrete but tightly linked structures. The bed nucleus of the stria terminals (BNST), central, basolateral and medial nuclei of the amygdala, the insulae and medial nucleus accumbens (NAc) form the hub of the EA [7, 8]. The EA forms a functional interface between the higher cognitive functions of the prefrontal cortex (PFC) and the cingulate cortex to the endocrine hypothalamus, the reward pathways of the striatum and to autonomic regulation from the brainstem to the peripheral nervous system (PNS). The EA is the seat of memory, emotion and motivational behaviors, fundamental to acute responses to danger and threat, fear, impulsivity, drug abuse and sexual drive. Functional disruption of the CRH in the EA is seen stress related psychiatric and somatic disease; major depressive disorder (MDD), generalized anxiety disorder (GAD), post-traumatic stress disorder (PTSD), “sickness syndrome,” chronic pain, cancer, metabolic syndrome, autoimmune conditions, other chronic medical disorders, substance abuse and addiction [6, 7]. Activation of neural circuits to acute stress occurs in micro to milliseconds. The resolution of current

technologies to tract rapid neurochemical events at this scale do not yet exist. Refinements in fMRI imaging of neural networks approaching this scale may become feasible at some point in the future. PET/ fMRI instruments may enable better correlations of neuroanatomic activation and functional neurochemistry though tracking events at this time scale will likely remain out of reach for several decades.

c) Anatomical map of CRH and CRHR1

CRH and CRHR1 show homology in regional expression; they are also found in PFC and frontal cortices, cingulate, insulae, the EA and hippocampus, the BNST and nuclei of the hypothalamus and brainstem. These areas are relevant to the behavioral and somatic adaptive response to stress. Stimulation or inhibition of the CRHR1 by CRH, related peptide analogues, non-peptide agonists or antagonists will precipitate or inhibit behavioral, neuroendocrine and sympathetic stress responses. Rodents show expression in areas primarily responsible for processing environmental stimuli, in sensory organ brain regions as noted above. Primate brains have limited concentrations of CRH and CRHR1 in these structures. There are differences in functional anatomy and circuitry with much lower receptor density and irCRH in sensory structures. Put in context, irCRH/CRHR1 appears to be more “front line” in direct stimulus processing (olfaction, auditory and visual cues) in rodents, whereas in primates neuronal projections from these stimulus processing areas are principally directed to areas with high density of CRH containing neurons that evoke cognitive, executive, emotive processing and social decision making. Limbic system regulation of lower order behaviors is similar between primates and rodents and relative irCRH/CRHR1 density and distribution remain comparable. Together CRH influences higher level integration of behaviors in more socially evolved species, while CRH modulation of autonomic processes remain more analogous to lower species. To date there is no specific radioligand for CRHR1; once developed the anatomic distribution and regional occupancy of CRHR1 would be of great benefit in understanding conditions of chronic and acute stress activation.

Acute Stress and Pathologic Changes from Chronic Activation

Survival is ultimate goal of all organisms and in humans and non-human primates (NHP); perception of danger both physical and social is critical to that end. Adaptations to these circumstances can be grouped grossly into fight, flight or freeze responses. Central regulation of the stress cascade directly or indirectly integrates multiple physiologic systems; disciplines such as neuro-endocrine-immunology are being recognized as critically important in somatic and central maintenance of homeostasis [6, 7, 10, 11]. CRH and its related peptides and receptors are many, tightly linked, act to direct physiologic actions often in contradictory directions under different and changing conditions of threat/ physiologic/environmental/psychosocial adversity. Rapid activation (seconds to minutes) of the systemic sympathetic adrenomedullary system releases catecholamines and stimulates visceral organs and vascular structures. As mentioned above, direct autonomic innervation and circulating catecholamines stimulate the heart, vascular structures, visceral and lymphoid tissues and increase cardiovascular, metabolic, immune, and other physiologic responses to stressful stimuli [6, 7]. This is a first line response.

The (dorsolateral PFC) dlPFC with the rest of the PFC is critical for a top down cognitive recognition of internal and external stimuli in ascertaining threat and an optimal behavioral response(s) is critical. The dense concentration and neuronal organization of irCRH/CRHR1 in the neocortical lamina and the co-expression of irCRH/CRHR1 with other neurotransmitters and neuropeptides imparts a multi-tiered neurochemical response. The mechanism(s) of cognitive recognition of danger and stress activation likely first engages the integrative areas of the PFC, the dlPFC and ventromedial PFC. These areas most densely express CRHR1 and irCRH. The processing of external information is a cascade of cognitive realization into consciousness which triggers memory and sensory input that the executive “mind” recognizes as threat or danger. Neurochemical network activation events occur in microseconds to seconds. Evidence for CRHR1 as crucial for this integrated process comes from studies in NHP treated with potent CRHR1 antagonists which mutes responses to acute social or environmental threats [12]. On the molecular and cellular level stress responses when acute are advantageous but sustained can be detrimental.

The PFC shows particular vulnerability to chronic stress induced injury. This is critical in understanding behavior; many research studies define stress mediated behaviors or have stress as a principal confounder. The PFC and dlPFC controls complex cognitive processes and those are disrupted in many brain disorders such as MDD, GAD, other affective disorders, addiction, attention deficit disorders, schizophrenia, PTSD, neurodegenerative diseases and others. The PFC and the dlPFC are integral in coordinating cognition and areas of the neocortex are most susceptible to degenerative and stress related damage. These disorders frequently find abnormalities in dlPFC and other areas of the PFC function on neurochemical, metabolic and anatomic imaging studies; parameters of chronic stress are frequent factors in disease vulnerability and pathogenesis [13, 14, 15, 16].

The effects of age, maturation, exposure to exogenous and endogenous environmental factors, genetic and epigenetic influences, the presence and duration of exposure to inflammation, adrenal and sex steroids as well as other stress hormones are critically important in maintaining prefrontal neuronal integrity. The stress cascade has a countless effects on neurochemical events and neuronal ultra-structure; genetic predispositions, epigenetic activation, deactivation or silencing of genes are poorly understood phenomenon. Dendrites appear to be highly sensitive to C and other adrenal steroids under conditions of chronic stress [14]. This can result in neuronal loss, dendritic regression and loss of connectivity in some areas while in other areas such as the amygdala, stress can promote neuronal hyperplasia and hypertrophy and dendritic expansion. Why stress targets one brain structure or loco-regional connectivity, a specific neuronal set, or axonal or dendritic cellular components is unknown. Neuronal connectivity within the neocortex and the PFC in particular has potent effects on the inhibitory and excitatory neurochemical balance in the top down control of distal circuits and their functionality. Stress toxicity could also favor hyperactivity in the amygdala which may enhance fear responses to environmental cues while decreasing neural inhibition in dlPFC and ventromedial PFC; chronically stressful conditions can fail to maintain effective executive control over limbic overdrive. Changes in dendritic connectivity may underlie diminished PFC activation and the pathologic, non-contextual hyper-activation of the amygdala seen in depression, neglect or PTSD [13, 14].

Whether neuronal injury is reversible varies by many factors including age, severity, duration of the stressor(s), the presence and sensitivity of glucorticoid and mineralocorticoid receptors, glucocorticoid concentrations, the anatomic structures involved and other elements in the neurochemical milieu. The impact of neurotoxic effects also depends on neuronal type, the type of exogenous or endogenous stressor(s), the presence of neural growth factors, its principal neurotransmitter and it position and action within the neural network [14].

Chronic activation of the stress system over weeks to many years occurs in a number of seemingly disparate disorders psychiatric, somatic (i.e. “sickness syndrome”) and both. Chronic medical disorders are often caused by inflammation, activated cellular immunity and cytokine release, loco-regional or systemic [6, 7, 17, 18]. Whether centrally and systemically generated, the chronic activation of extra-hypothalamic CRH and the (HPA) drives excess GC/cortisol release can determine the severity of illness. Resultant hypercortisolemia diminishes feedback responsivity throughout the CNS and other stress sensitive organs, causing glucocorticoid resistance and diminished GC tissue sensitivity leading to immune and neurotransmitter dysfunction [10, 11].

Proximal and Distal CNS Network Regulation

Understanding the complexities of stress system neuroanatomy and the inter-regulation of the irCRH/CRHR1 neuronal expression and neuronal projections to other critical neurotransmitters have been limiting in understand stress in primate central nervous system. Figure 2 shows a simplified diagram of a few of the interactions between cortical and subcortical structure and their influence on stress hormone and neurotransmitters actions.

Early work with irCRH and CRHR1 was able to show the relative regional expression in the CNS in rodents and non-human primate NHP [5, 19]. Qualitative molecular studies were critical to the early understanding of the interface of CRH and the structures involved in stress regulation and gave insights into the regulatory networks.

Evolving technologies and studies of optogenetics in rodents have greatly expanded our knowledge of the neural networks associated with stress activation. Optogenetic techniques are recent developments and involve introducing light sensitive proteins into cells using viral vectors which when exposed to specific frequencies of light can trigger cell firing or silencing and localization. Targeting photosensitivity to specific cell types (neurochemical expression) enables tracking of those cells through their networks to better elucidate loco-regional connectivity. These studies have been principally focused on subcortical structures in rodents; these techniques have increased our basic understanding of the interface of critical anatomic and neurochemical control hubs. This technology continues to emerge and optogenetics has guided understanding into the greater complexities of the primate brain [20, 21].

From these studies we learn a key structure in stress signaling is the BNST; it serves at central link between amygdalar complex and the dopamine (DA) concentrated reward centers of the striatum, ventral tegmental area and the NAc. Critical also is connectivity to hypothalamic and midbrain areas which relay stress control over the HPA, other

neuroendocrine hormones and the SNS. Excess CRH expression in the BNST and its projections drive many stress behaviors; in rodents they can be stereotypic, model driven, well characterized and reproducible. In primates network activations are more complex, driven from and to limbic structures and neocortical areas especially the multiple PFC bundles, the insulae and anterior cingulate cortex (aCC). Clinical studies find amygdala activaton and PFC inhibition with fMRI in a number of psychicatric disorders including PTSD, GAD, maternal deprivation (MD) and MDD [22 – 26]. Seemingly disparate disorders share network and structural connectivity but they lack specificity; making clinical utilization of these functional technologies nonspecific at this point in their development [20, 27, 28, 29].

Genetics/Epigenetics

Important to this end is the work from the Oregon Primate Center [31]. Their studies of stress on reproductive functions in female macaques have helped develop a neuroanatomic and neurochemical understanding of stress sensitive (SS) and stress resistant (SR) individuals. The investigators were able to induce behavioral and neurochemical trait markers into NHP. These are genetically/epigenetically stable and enable delineation of the susceptibility of individuals to changes in environmentally stressful state conditions.

They have shown that both vulnerability and stress exposure controls expression of irCRH and serotonin (5HT) in regulatory sites such in the entorhinal cortex (ERC) and brain stem nuclei i.e. dorsal raphe (DR), LC. Exposure to graded stressors with measured “clinical” outcomes will modulate expression of irCRH, CRHR1, 5HT receptors, transporter and 5HT metabolic enzymes.

Their studies suggest that stress responsivity is both a state and trait condition; as shown SS when compared to SR NHP show fundamental differences in responses to stressful stimuli *de novo*. Correlations between behavior and 5HT and CRH expression are predisposing traits which in stress trigger further neuropathologic changes [32]. irCRH neuronal expression conveys stress sensitivity throughout multiple neuronal networks; they show integrated yet redundant pathways that link the neocortex, limbic, striatum and brainstem. Major stress conductive pathways originate in the PFC layers III and V which project to the ERC, insulae, aCC, temporal lobes and to other PFC bundles; additional irCRH projections from PFC go to amygdala and parahippocampal structures. irCRH and 5HT neurons originate in brain stem nuclei and transit rostrally to afore mentioned stress susceptible networks and contribute to both top down and bottom up signaling.

Associated co-expression of irCRH with 5HT receptors, transporter (5HTT), 5HT metabolic enzymes in SS/SR show integrative functions of these to regulate stress responsivity. These data strongly support many rodent studies showing interaction of 5HT, *gamma*-aminobutyric acid (GABA) and CRH control of acute and chronic stress. These findings may determine trait and/or state vulnerability to stress sensitivity and resistance [33]. They show differential neurochemical and anatomic changes in SS/SR based on cell density and molecular expression associated with phenotypic stress resistance or sensitivity [34, 35]. These highly

complex but expected patterns of integration are consistent with our limited imaging signals when viewed en mass.

Environmental and genetic influences have profound effects on stress responsivity; these can manifest at different stages of life, varying pharmacologic exposures, and are altered by changing physiologic milieus and behavioral events [13, 14, 36]. Hsu et al., found the minor allelic differences in the CRHR1 gene effects on blood oxygenation level dependent (BOLD) fMRI activation patterns in normal individuals carrying the A-allele in targeted limbic areas when compared with normals carrying the at risk GG genotype [37]. The increased BOLD signal in the medial temporal lobe is ascribed as protective in emotional processing of negatively loaded stimuli. These findings point to a genetic predisposition to vulnerability to MDD but they do not exclude the presence or absence of earlier life events as epigenetic modifiers of phenotypic expression.

The early maturation period is very sensitive to stress mediated developmental changes on an anatomic and neurochemical level in NHP [38, 39]. Previously institutionalized children who suffered from MD were studied for stress activation and found evidence of neuronal network maturation as a function of early life stress exposures. Children who experienced MD had earlier maturation of amygdala-prefrontal cortical connectivity compared with children without early life adversity.

C activation was also increased in response to the stress of the experimental procedures. Amygdala activation patterns were elevated in the MD group though overall anxiety measures were lower. These results suggests the adverse environment and conditioning leads to early maturation of neural networks which mitigate other abnormalities in limbic function i.e. a hyperactive amygdala, with greater top down anxiety suppression by prematurely developed PFC/limbic connectivity. However the exaggerated stimulatory effects of the SNS and hypothalamus remain intact causing stress activation and chronically increased C. Combined overactive stress activation with circuity potentially lacking plasticity necessary for future adaptation may contribute to pathologic responses of the amygdala to future stressors. They report the potential for future vulnerability from early network development that may limit the emotional repertoire and intellectual development necessary in adulthood [24].

GCr is highly concentrated in the medial temporal lobe involving most of the structures of the EA critical for limbic emotive regulation and fight, flight or freeze reactions as well as memory retrieval and consolidation. These receptors are also highly expressed throughout the dlPFC, medial and lateral regions of the prefrontal cortex. Activation and deactivation of specific genes responsible for cognition, memory and stress activation show long term patterned responsivity by the steroid milieu.

HPA activation results in elevation of circulating C which shows affinity in primate brain to glucocorticoid receptor (GCr); increases and abrupt elevations in C concentrations modulates short and longer term neuronal function. C through the GCr generates genomic activation through internalization of a GC/GCr and nuclear translocation. DNA is encased by chromatin which is comprised of tightly wrapped complex histone protein octamers, the

nucleosomes. Sequentially arranged nucleosomes make the chromatin structures of the chromosome. Histone chemistry is controlled by target enzymes which cause histone methylation/demethylation and acetylation/deacetylation. These enzymes change the histone protein electrostatic state; the actions of DNA demethylase and histone acetyl transferase can uncouple histone proteins to allow transcription of target genes; methylation or deacetylation of histones will block access to genomic regions causing deactivation or silencing. These proteins comprise a composite regulatory system managing the availability of DNA to be transcribed into RNA for synthesis of proteins and other gene regulatory factors (Figure 3).

Epigenetic changes in the CNS in regard to stress activation, GC/C and GC resistance are incompletely understood. Delineation of epigenetic mechanisms within the CNS *in vivo* awaits substantial refinements in pharmacology, surrogate markers of gene expression and imaging technologies. *In vivo* imaging of second messenger and related gene/receptor expression in oncology may allow future applications in neuroscience [40, 41, 42].

Molecular Imaging

Neurochemical/Neuroreceptor Imaging

Serotonin (5HT)—Direct application of CRH into DR suppresses 5HT release in preclinical models of GAD/MDD. CRH is co-localized on 5HT and GABA neurons. CRH stimulation of GABA suppresses 5HT activity which in turn can drive stress activation in central nucleus of the amygdala. The relationship between 5HT and CRH is both state and trait dependent as was noted in the SS and SR NHP [31]. The state of the stress axis and central CRH activation in the setting of chronic stress is associated with MD in rhesus PFC which shows increased cerebellar, dorsomedial PFC (dmPFC), and aCC volumes. When Spinelli with others tested for 5HT1a receptor density with ^{18}F-WAY they found generalized decreased receptor distribution in all areas tested except the dmPFC in females [38, 39]. This finding in NHP is consistent with autopsy studies in depression that shows some but not all 5HT receptors are involved; neglect and other developmental insults are not readily amenable to prospective neurochemical imaging studies and assessment of past life experience can be flawed [44]. Many radiotracers have been developed and used for 5HT PET studies; Paterson et al. provide a comprehensive review of current and future compounds and their applications [44].

Dopamine—Induction of the acute stress response by administration of peptide CRH into the CNS (i.e. microdialysis) can mimic acute behavioral stressors. This has been shown to trigger an abrupt DA release. This has also been seen in clinical studies with raclopride (RAC), a specific agonist radiotracer for the DA type 2 receptor, displacement PET imaging in response to a wide variety of behavioral and physiologic stressors [46, 47]. DA has dual roles, activation and release in response to real and anticipated reward in striatal reward centers in the striatum and NAc as well as to aversive stressors [48, 49]. This has been shown in patients, substance abusers and naïve controls [50]. Findings are influenced by chronic substance abuse, psychotic and affective disorders though commonalties exist. Stress mediated DA release is seen in cognitive areas of the prefrontal cortex in controls [50]. fMRI BOLD activation in amygdala and cingulate in response to negative affective

stressor stimuli finds correlative dependent release of 6- [(^{18})F]fluoro-L-DOPA (dopamine surrogate) on PET imaging. Increased activation which is associated with fear and threat reactions accentuates DA release. This suggests a modulatory effect of negative behavioral affect on DA [51]. Stress induced BOLD signal in the NAc correlates with C response and this is interpreted by Oei et al to represent DA release in nonsubstance abusing men [52]. The impact of state conditioning on NAc and presumed DA activation implies an association of behavioral cues to DA as a neurochemical marker of stress modulation; taken together the preclinical supports clinical observations of similar neurochemistry and neural networks modulating stress, DA and reward.

Glutamate—Behavioral models of stress and anxiety show that exposure to stress provoking environmental stimuli can elicit glutamate (GLU) release in target brain regions associated with stress responsive behaviors [53]. The molecular actions CRH, CRHR1 and GLU show tight integration in studies by Refojo et al. Genetic knock-out and knock-in techniques were used to identify GLU on CRH containing neurons throughout the rodent CNS and they show stress associated behaviors related to CRH and GLU activation [54].

Glutamate and its metabolite glutamine can be identified and quantified on pMRS (figure 4) along with other neurotransmitters, membrane elements and metabolic markers. New radiotracers are being developed for the multiple GLU receptors and transporters [55]. As a potent excitatory neurotransmitter GLU has modulatory effects on DA and has actions in striatum and reward pathways. Clinical studies on metabotropic GLU receptor subtype 5 (mGluR5) report GLU differences in cocaine and nicotine addiction and other psychiatric disorders [56 – 59]. GLU is recognized as integral in modulating other neurotransmitters and neuropeptides [60].

DeLorenzo et al. examined PET scan test re-test binding variability using the mGluR5 ligand, [(^{11})C]ABP688, between subjects and found increased binding on the second of two scans in 7 of 8 participants. This was not observed in anesthetized baboons. Having controlled for other parameters (i.e. mass dose, imaging time, specific activity, tracer dose) and having found no difference in metabolism or other systematic technical problems. The possibility of stress response to the novelty and unfamiliarity of the first PET procedures compared with the second was raised as a consistent variable and possible confounder. No hormonal or behavioral measures were obtained so this is speculative but this raises an interesting possibility and line of investigation especially in neurochemical systems that are highly stress sensitive [59]. Between the preclinical and clinical observations it appears that GLU, mGluR5 and other GLU subtype receptors may be stress sensitive and thus activation could be both a valuable marker and significant confounder in future investigations [60]. Multiple GLU ligand markers are under development (figure 5).

***Gamma*-aminobutyric acid (GABA)—**CRH activates *gamma*-aminobutyric acid and at physiologic concentrations GABA stimulation can inhibit 5HT in DR and terminal 5HT projections. This is thought to contribute the development of chronic stress conditions and may determine trait/state vulnerability to SS/SR [33]. The infusion (microdialysis) of CRH in CeA increases GABA in control and alcohol (ETOH) dependent rats though the CRH effect on GABA was muted in ETOH dependent animals [61]. C.J. Cook exposed

unrestrained sheep to a dog threat in a microdialysis experiment and found a relationship between cognitive recognition of threat by the sheep with release of CRH from hypothalamus, adrenal C secretion and co-release of GABA from the amygdala. This suggests that the acute inhibitory effect of limbic GABA release generates a stimulatory action on CRH release and subsequent HPA activation [62].

Chronic PTSD is associated with persistent fear and anxiety with chronic stress activation. Inability to inhibit fear related memories and associations are pathognomonic to the disorder. GABA co-regulates glucocorticoids (GC) and catechols with hypothalamic CRH and pituitary ACTH. Decreased GABA type 2 receptor binding has been described. PFC and areas of the EA are principally affected and represent CRH mediated neural networks [63, 64].

pMRS can identify a broad spectra CNS neurotransmitters, their metabolites, cell structure molecules, energetic compounds and their byproducts (figure 6). GABA has a target signal and PTSD patients in a pMRS study of insulae metabolites were found to have a significantly lower metabolic GABA signal interpreted as a deficiency in inhibitory GABA neurochemical state; this may explain the clinically permissive activation of PTSD memories and recurrence of invasive recall symptoms [65]. Deficiencies in GABA have been reported in schizophrenia and other disorders [66].

Norepinephrine—The development of highly specific PET ligands for norepinephrine transporter (NET) has lagged behind those for DAT (DA transporter) and 5HTT and other receptors. NE is a critical neurotransmitter for the modulation of the HPA and stress mediated behaviors [67]. NET unlike 5HTT and DAT are highly localized within discrete brainstem nuclei, hypothalamus, thalamus and subcortical structures. In recent years clinical studies relevant to stress and stress related conditions have been published. [^{11}C]methylreboxetine has been recently tested and found to have suitable specificity and early studies have been completed (figure 7). Two recent studies one on post-traumatic stress disorder (PTSD) and another on cocaine abuse demonstrate under the conditions of chronic stress from these disorders show that NET density shows significant differences compared with controls. In PTSD moderate to marked reductions in NET are seen in patients while in cocaine abusers significant increases (age corrected) were seen in nearly all regions tested. Interestingly the magnitude of the differences are comparable between groups though in opposite directions [68, 69]. These studies demonstrate that chronic stress related conditions effect NET density. What is unclear are the effects of acute or sub-chronic stressors; metabolic challenges such as insulin hypoglycemia and other conditions/agents activate the SNS and adrenal medullary release of both norepinephrine and epinephrine. The time course and potential effect(s) of acute stress responses on NE release and changes in binding potential (BP) or rapid shifts in NET B_{max} is unknown [70].

Neuropeptides

Substance P/Neurokinin1—Stress triggers substance P (SP)/Neurokinin1 (NK-1) release; SP/NK-1 is the ligand for and acts through the neurokinin1 receptor (NK1r). Acute stressors can elicit release of SP in structures throughout the EA, striatum, the DR, and LC

[71]. Specific antagonists for SP at the NK1r will both decrease HPA reactivity and stress induced behavior. Some are associated with increased 5HT transmission, efflux, release and activation of 5HT1a. 5HT1a antagonists blocked both behavioral and hormonal effects at NK1r [72]. Stress activation through HPA and C release will modulate SP in EA other than at 5HT1a suggesting multiple pathways for modulation of 5HT under behavioral/physical stress. Associated with behavioral disorders, SP/NK-1 is under development as an anti-depressant with several candidate compounds showing efficacy in clinical trials [73]. PET studies have shown decreased NK1r BP changes in patient groups and numerous PET ligands have been developed and are showing utility for drug development [74, 75].

Orexin/Hypocertin—Orexin/Hypocertin (O/H) is tightly linked to extra-hypothalamic CRH neuronal networks and may play an adjunctive role in stress activation and response to aversive conditions [76]. irCRH is co-expressed in hypothalamus with O/H neurons which project to PFC, hippocampus, DR, LC, ventral tegmental area (VTA) all critical to CRH activation; activation of the O/H neurons in chronic stress states such as cocaine and opiate dependence can contribute to drug reinstatement and relapse. O/H activation through its projections to VTA has marked effects on DA release as a secondary stress effect and DA release is a pathognomonic of addiction [77, 78]. No *in vivo* PET imaging agents for O/H are available in humans at this time though several are under development [79].

Nociceptin/Orphanin FQ—Nociceptin/Orphanin FQ (N/OFQ) is an atypical opiate peptide which has been demonstrated to have antagonistic effects on CRH in the amygdala. Administration of N/OFQ in DR can antagonize the neurochemical induced stress effect from CRH administration and behavioral stress from forced swim tests with concomitant effects on 5HT and GABA release. Though understanding of it is incomplete this neuropeptide has been observed to curb CRH actions in other stress related territories [80, 81]. A new positron labeled N/OFQ ligand has recently been described for *in vivo* imaging in humans (figure 8); the impact of stress and stress activation from N/OFQ remains to be elucidated [82].

Endorphins and related peptides—Conditions of chronic stress and addiction from cocaine and alcohol show differential changes in beta-endorphin (END) measured with ^{11}C carfentanil (CFN) PET BP at baseline and with provoked craving [83–85]. Acute pain can release END as measured w/CFN PET displacement studies [86]. Many other provocative stimuli can elicit END release. Though a fundamental problem with all CFN studies are confounders related to differential expression and affinity of the mu receptor vs. END release and their relative contribution to the BP signal.

Acute stress activation i.e. to fear or novelty has not been tested but HPA activation is suggestive of a central CRH/ACTH response with possible END and CFN effects. The mu receptor gene shows different pharmacologic and behavioral responses and CFN has shown subtle BP differences. Decreased striatal BP with CFN PET correlates with increased central HPA reactivity and C release to naltrexone mu receptor blockade indicating central regulatory action of END and perhaps related opioid peptides to the stress system [87].

Functional Imaging

Neurocognition and Stress

fMRI evaluates regional neuronal activation due to changes in BOLD in response to variable cognitive and physical stimuli. This technology is limited by the constraints of the experimental design, the physical limitations of the MRI scanner and the physics of high strength magnetic fields.

Experiments that induce fMRI stress activation are nearly countless. Tasks and paradigms have been developed to test brain BOLD patterns highlighting psychiatric disorders, i.e. MDD, other affective disorders, schizophrenia, PTSD, drug interactions, drug abuse, relapse and vulnerability to licit and illicit substance use. Confounders include but are not limited to additional co-morbid conditions, gender, age, education, intellectual capacity, medication and drug exposures, language, handedness, head trauma, differences in neuroendocrine homeostasis. Likewise the number of fMRI studies that have studied stress reactivity on cognition, anatomic variability, emotional processing and reactivity, memory, decision making, craving and others, easily number into the hundreds[88 – 96].

CRHR1/CRH in EA, PFC, DLPFC, amygdala, striatum and hippocampus process anxiety, fear, and memory consolidation all of which assign emotional salience when presented with fMRI tasks involving fear, anxiety, decision making and reward. The commonality of the circuitry defines the emotional condition and conditioning of the individual during any procedure or task; the procedures impact and the performance is impacted by whether the emotional circuitry is engaged in an underlying state of activation. BOLD signal in networks activated by CRH and CRHR1 may set the signal “floor” or “ceiling.”

Stress activation with Tier Social Stress Test (TSST) induces increases in C and NAc activation compared with a non-stressed control activity. This increase was graded with the C response from the TSST stress stimulus when compared to the non-stressed control condition. This NAc activation was assumed to correlate to DA release as has been described with stress and HPA activation [51]. Oler et al., (2009) studied stress susceptibility with in NHP with anxious temperament with glucose metabolism using [^{18}F] fluoro-2-deoxy-D-glucose (FDG) PET in response to separation stress [97]. Significant increases in glucose metabolism were observed in these animals in the EA and correlative analysis found the increases in metabolism were associated with magnitude of observed behavioral stress.

HPA activation results in elevation of circulating C which shows affinity in primate brain to glucocorticoid receptor (GCr); increases and elevations in C concentrations modulates short and longer term neuronal function. C through the GCr generates genomic activation through internalization of a GC/GCr and nuclear translocation. DNA is encased by chromatin which is comprised of tightly wrapped complex histone protein octamers, the nucleosomes. Sequentially arranged nucleosomes make the chromatin structures of the chromosome susceptible to changes in histone target enzyme action with subsequent methylation/ demethylation and acetylation/deacetylation events.

GCr is highly concentrated in the medial temporal lobe involving most of the structures of the EA critical for limbic emotive regulation and fight, flight or freeze reactions as well as memory retrieval and consolidation. These receptors are also highly expressed throughout the dlPFC medial and lateral regions of the prefrontal cortex. Specific genes responsible for cognition, memory and stress activation show long term patterned responsivity by the steroid milieu.

Henckens et al. studied volunteers with fMRI and found that exposures to physiologic levels of oral C administered three hours prior to memory testing improved task performance that was associated with increases in BOLD signal in dlPFC. Acute exposure to oral C at 30 minutes, a time course that could mimic HPA activation in a fight or flight situation did not affect task performance or dlPFC BOLD signal. Temporal delay in memory improvement on task corresponds to preclinical data which suggests that epigenomic as well as neurochemical actions on GCr sensitive neurons (and possibly other cellular elements) may be necessary for memory consolidation from stress [98].

C through the GCr modulates GLU and these effects appear to be of the same time course as those seen with the cognitive improvements and purported genomic activation of C exposure. This supports delayed alternate neurochemical/neurotransmitter action(s) from C exposure in addition to those activated acutely in the acute stress response i.e. epinephrine, norepinephrine [99 – 101].

These observations suggests that exposure to stressors can impact cognitive performance and issues such as novelty and anticipatory effects on mood can change ability to execute tasks, influence test, retest reliability of cognitive testing and differentially effect neurochemical studies with a variety of neuroreceptor agents i.e. DA, mGluR5, 5HT receptor specific agents. .

Neuroanatomic MRI/DTI

Diffusion tensor imaging (DTI) can characterize microstructural alterations in white matter; the ability of water (easily detected on MR) to diffuse in white matter tissues gives an index of the organization and structural integrity of glial tissues and myelinated axons tracts which correlates with the regional brain connectivity. Conditions of neurodegeneration, brain injury, psychiatric disorders and toxic exposure can result in microstructural white matter tracts damage. Figure 9 shows schematic representation of white matter tracts from spinal cord into subcortical and cortical brain regions. PTSD and alcohol abuse are frequent comorbid conditions both associated with chronic stress system activation. Measuring white matter integrity with diffusion tensor imaging finds decreased fractional anisotropy (FA), a measure of decreased white matter neuronal tract integrity in patients with PTSD independent of alcohol abuse [102].

Combat soldiers developed cognitive, structural white matter changes and reductions in functional conductivity when pre and post service fMRI/DTI studies were compared. However no abnormalities were evident in never deployed soldiers. No participant in either group developed PTSD or met diagnostic criteria for psychiatric diagnoses prior to or after deployment. No group changes were detected between groups at baseline. Cognitive

changes were observed with attention and memory deficits as were decreases in FA/ decreased white matter integrity seen upon short term follow up in those post combat. The largest decreases were associated with the greatest decreases in executive functioning. Of particular concern was that after longer term evaluation at 1.5 years some areas of white matter, midbrain/prefrontal linkage did not revert to baseline integrity, which brings to question vulnerability to future stress mediated illness and cognitive dysfunction. Also amygdala frontal connections appeared to have suffered longer term changes which raise possible future stress susceptibilities [103].

Another cross sectional investigation of survivors of the recent Japanese earthquake in 2011 found that decreased FA was associated with higher anxiety in individuals not meeting criteria for PTSD. In addition there was increased disorganization of tract architecture when pre and post-earthquake disaster patient prospective studies were compared. Does the initial observation of decreased FA represent a trait marker for anxiety or result of cumulative exogenous stressors i.e. genetic predisposition or epigenetic transformations of neural tracts? And even in individuals who experienced severe distress after a monumental natural disaster but recover and don't develop chronic traumatic psychological injury still show disruption of neuronal pathways. These data point to the potential for CNS neuroanatomic disruption from "subclinical" stress exposures [104, 105].

Integrating Multimodality Imaging Technologies

Neuroendocrine and Metabolic Influences on Imaging Stress, Reward and Behavior

Metabolic effects on DA release with RAC shows correlation with circulating leptin, an adipose endocrine hormone with central CNS, peripheral endocrine and local paracrine actions in adipose tissues. Also studied were the effects of allelic differences in leptin genes on RAC BP and they found sensitivity of DA release to stress provocation influenced by different leptin alleles [98]. This is consistent with low dopamine seen in obesity, higher BMI, high fat dietary composition and factors related to metabolic syndrome. Leptin modulates feeding behaviors and the homeostatic metabolic state; it has effects on recognition of salient responses to food cues and stress mediated behaviors. Basal metabolic state, leptin concentrations, presence of insulin resistance, body mass index (BMI) and appetite stimulation can alter DA release. Exogenous stressors within or independent of a study paradigm may have influenced study results. Elevation of BMI can have independent effects on limbic activation and is associated with chronic stress and hypercortisolemia; presentation of palatable foods in the context of acute stressors can potentiate limbic activation especially the amygdala and insulae as well as increase vulnerability to stress induced non-homeostatic eating and obesity [106 – 108]. Genetic and epigenetic influences and the remarkable redundancy of CNS feeding mechanisms throw considerable uncertainty into the interpretation of imaging results.

Overweight women with insulin resistant polycystic ovary syndrome (IR-PCOS) and healthy normal weight otherwise matched controls were tested with both PET for mu opiate receptor BP using CFN and fMRI. CFN BP and release is interpreted as a marker of motivational behavioral rewards. The IR-PCOS group received short term treatment with metformin an anti-diabetic, anti-insulin resistance medication. When faced with stressful

emotional tasks the IR-PCOS they showed greater activation on fMRI and mu receptors (higher BP) in emotionally salient regions of the EA and associated neocortical areas (aCC, MPFC [medial PFC], striatum) when groups were compared [101]. Mu opiate BP normalized to control concentrations with medication treatment. The IR group showed significant improvements in positive affect and CNS activation on fMRI responses to the emotional stressors despite failing to achieve weight loss and significant improvements in clinical and biochemical markers of IR [102].

Nora Volkow, an expert in addiction, points out that stress network circuitry in food intake, obesity and its subsequent metabolic effects have neurochemical underpinnings with other motivational disorders and these changes correspond to activation and hormonal patterns in chronic stress conditions. This suggests a basic dyshomeostasis over a considerable range of BMI and metabolic conditions and presents a variable in studying network activation patterns [109 – 112].

Potential Stress Impacts on Imaging

As noted from NHP studies both state and trait stress resistance or sensitivity can have considerable influence on the expression on both 5HT receptors and 5HTT. They also showed that treatment with serotonin selective reuptake inhibitors (SSRI) had no influence on 5HT receptor expression in key target regulatory areas [34, 113]. This finding points to the durability of genetic/epigenetic factors and stress in determining the neurochemical foundation of individuals and other stress related variables for imagers to consider.

In a comprehensive review of PET studies in depression, Savitz and Drevets (2013) report that HPA activation with acute stress steroid release can effect 5HT neural activity and chronic activation in the setting of repeated stressors with elevated GC can reduce 5HT1a density [30, 114 – 116]. 5HT2a receptors also show significant stress and CRH sensitivity. Acute activation of hypothalamic 5HT2a receptors can reciprocally stimulate the HPA. Chronic stress with elevated C has been observed to increase 5HT2a binding potential. Trait characteristics such as anxious temperament in NHP has been associated with increased 5HTT BP [96].

Depressed patients with increased stress activation tend to have overall higher 5HTT density in limbic regions and this may be a trait marker for disease and future stress mediated exacerbations of depression. Savitz and Drevets conclude that serotonin based functional imaging is a valuable tool in understanding depressive disorders. Susceptibility to stress, the presence of chronic stress and even diurnal variations in stress hormones may have direct effect on the BP of 5HT markers *in vivo* both in patient *and* normal comparison groups

As noted by DeLorenzo in their reproducibility study the presence of test novelty might have significantly influenced mGluR5 binding. How novelty might impact stress or CRH activation and mGluR5 are unknown; firm demonstration of fact requires both hormonal and behavioral measures but these variables are rarely considered in any imaging study unless specifically addressed in the hypothesis [58]. This reinforces the potential complexity and confounds posed by stress activation.

Even transient changes in the stress milieu with physiologic adrenal C release activate areas critical for cognition and memory. The temporal course of memory consolidation underlies a myriad of interactions in gene activation/deactivation, circuitry and neurochemistry. The timing of cognitive challenges and even the participants' arrival time prior to an experiment may influence memory and emotional salience given C effects on gene transcription and expression of new memory encoding [98].

CRH Receptors as a Molecular Target for Imaging

High potency CRHR1 antagonists have been a focus of pharmaceutical industry for nearly 2 decades. Many clinical trials have been performed and despite evidence of pharmacologic actions of CRHR1 in clinical trials, pharmacologic and imaging studies; no phase III study has demonstrated a clear advantage or clinical benefit to this class of agents when compared to active or inactive placebo. There are many hypotheses for these results ranging from clinical trial design and patient selection to low antagonist action at the CRHR1 *in vivo* [117, 118].

The first study in 2000 by Zobel et al., found that CRHR1 antagonist R121919 had clinical efficacy in an open label trial in patients with moderate severity MDD [119]. Despite early promise CRH antagonists were limited to industry sponsored clinical trials and experimental paradigms. Their failure to show effectiveness in patient populations with MDD, GAD, irritable bowel syndrome (IBS) curtailed further development of these agents Despite the failure of clinical trials, a CRHR1 functional imaging agent may prove valuable to better stratify diseases hereto not well understood (i.e. depression, PTSD, IBS, neuroimmune conditions). Also an *in vivo* imaging agent may elucidate the activation of the stress cascade and other physiologic systems.

At least 4 imaging groups have attempted development of radiolabeled CRHR1 ligands. Physiochemical properties necessary for CNS imaging ligands include crossing the BBB, limited tracer metabolism, high target affinity given the unique molecular properties of the CRHR1 as well as receptor density [117, 118, 124 – 127]. Several reported compounds had physiochemical properties *in vitro* but none demonstrated *in vivo* binding in nonhuman primates. Using functional imaging agents can aid drug development; enabling delineation of receptor concentration and occupancy needed for clinical effect. They may prove diagnostic in patients with "stress" vs. "non-stress "conditions.

Several groups have used advanced neuroimaging to extend behavioral observations to discern if these agents will generate a CNS signal relevant to behavioral and somatic indications for the drugs [125 – 129].

Hubbard, Labus with others studied IBS which causes chronic, unpredictable and often intractable episodes of abdominal pain with changes in bowel function. In many, IBS leads to a syndrome of conditioned fear. They postulated that regional circuits modulating conditioned fear responses would show heightened activation in response to aversive pain conditioning and would be resistant to deactivation during extinction the experimental procedures. Testing both patients and controls with fMRI administration of proprietary CRHR1 antagonist GW876008 (GlaxoSmithKline) diminished activation of these regions in

both groups though with their limited differences during the acquisition phase of pain stimuli in all. Reduced activation of the thalamus in IBS during pain suggests diminished pain appreciation [125, 126].

Once the pattern of pain expectancy was established, extinguishing pain showed profound differences between patients and controls. Those with IBS when treated with placebo had persistent fear circuitry activation in marked contrast to the control volunteers. With GW876008 the IBS group showed more suppression and deactivation of the CNS circuits than controls.

Hypervigilance, conditioned fear and anticipatory anxiety show commonalities with other stress mediated conditions such as GAD and PTSD. These disorders have similar deficits in fear conditioning and extinction learning after aversive stimuli is withdrawn. The failure to inhibit fear appears to be a cardinal feature of these disorders; the CRHR1 antagonists show efficacy in facilitating extinction learning. These findings suggest that stress activation is critical for both the acquisition and extinction of aversive events. The brain regions involved show high expression of CRH and CRHR1 and are blocked by the antagonist. Activation and neural circuitry patterns seen share considerable overlap with the activation patterns seen in PTSD and GAD memory reactivation [125, 126].

Administration of proprietary CRHR1 antagonist R317573 (Johnson and Johnson) found that at low and high doses reduced regional FDG metabolism (rCMglu) in areas known to activate anxiety i.e. amygdala, hippocampus, striatum while neocortical areas showed increases in regional glucose measurements i.e. rCMglu. These areas are behaviorally relevant to mood and anxiety disorders and are also known to have high concentrations of CRHR1 (127). Neural circuitry affected by this antagonist has been well described and are consistent with the fMRI findings of Hubbard and Labus [126].

fMRI and FDG PET studies show structural and activation patterns from CRH/CRHR1 drugs but delineation of specific neurochemical inhibitory/excitatory events cannot be extrapolated from these data. CRH has known neurochemical and physiologic regulatory actions throughout these structures. Integrating anatomical neural activation patterns with stress responsivity and CRH/CRHR1 neurochemistry await specific *in vivo* markers.

Discussion

It often seems biological psychiatry has largely subsumed the fields within functional imaging. That is not to say that a majority of functional imaging and its releavnce to the stress system is directed to the study of the brain. Stress mediated illness both psychiatic and medical is very common as noted by Chrousos GP [6, 7]. Stress activation is a component of a broader picture of pathophysiology and resultant clinical outcomes. Table 1 shows functional imaging targets/techniques that have/may show empiric responses to the presence of stressful stimuli.

Accounting for stress should increase the sensitivity and specificity of disease detection and classification and improve intervention effect size. As noted by Kapur S et al. delineation of patients with common disorders from healthy individuals poses little diagnostic problem.

Differentiating phenotypically similar disorders can be difficult. Decisions based on incomplete information may result in selection of incorrect medications or therapeutic interventions and diagnostic errors lead to treatment failures, unnecessary side effects and additional costs [130].

This is also relevant as the cost of development of new CNS medications continues escalate with time from discovery to market being about 13 years which is up to 35% longer than non-CNS drugs. Even more alarming is that of 100 candidate CNS molecules 8.2% reach Phase I clinical trials (compared with 15%) and only 46% survive Phase III compared with 66% of non-CNS candidates [131, 132, 133]. Several pharmaceutical companies have already discontinued medication development with others actively considering the same; reasons include delays in approval processes, costs of clinical trials, low yield to reach successful completion of Phase III, failure to demonstrate substantial clinical benefit over existing available agents and post marketing liability.

Developing more cost effective measures to aid development of medications is critical to stem increased expenditures and delays for needed medications. Sensitive biomarkers have potential to facilitate discovery with both individual and societal benefits. Demonstrating that new medications generate a CNS signal with imaging using relevant test markers, have favorable CNS penetration, pharmacokinetics, bioavailability and receptor occupancy can eliminate some candidates earlier in development cycle. No one single piece but several likely will enable an earlier “go, no-go decision.” Streamed line approvals for micro dose imaging studies through a more rapid FDA exploratory IND mechanism can reduce the overall time in the pipeline.

Diagnostic measures for many CNS disorders such as affective disorders lack specificity; given the state of knowledge and fundamental commonalities of biologic markers available to date, segregating criteria are limited. Adding more upstream markers such as glucocorticoid receptor markers, neuropeptide ligands and developing correlative analysis for multimodal imaging may enhance decision accuracy for clinicians. Increasing integrating different imaging modalities to address questions of the relationship of cognitive activation with neurochemical markers are being used i.e. neurochemical and metabolic PET with fMRI and pMRS [50, 109, 110, 113]. Knowledge of the state of the stress system is crucial to improve diagnostic specificity and importantly the effect size.

We show acute and chronic stress activation through CRH/CRHR1 has direct and indirect neurochemical and neuroanatomic effects on CNS systems reflected in clinical disorders. Mostly by inference and increasingly by direct observation and assessment of data the stress system regulates neurochemistry and neural networks. Chronic stress can lead to lifelong genetic/epigenetic changes through gene activation and deactivation which can involve increased CRH expression and C overproduction. Genetic predispositions show that individuals have inherent stress vulnerabilities and dependent on environmental conditions may or may not manifest them.

Chronic stress invokes somatic and neurotoxic effects and high resolution anatomic imaging can now better delineate these effects. Questions regarding reversibility and regression are

unclear with many influencing factors both exogenous and exogenous unique to the individual. Co-localization in cells and networks infer CRH/CRHR1 importance; higher cognitive areas that express CRH/CRHR1 are often impaired. The sensitivity of advancing imaging technologies increases the ability to discern functional abnormalities in individuals that fail to meet diagnostic criteria for clinical disorders and/or prior to the onset of symptoms. Advances in molecular imaging have found correlates with specific neurotransmitters and clinical disorders but the consistency of these findings and their practical applications are limited. Despite this molecular imaging has revealed insight into drug action and perhaps in the future may predict medication responses.

The relative lack of basic understanding of the CNS in both health and disease is further hampered by preclinical models with limited predictive value when translated from bench to bedside. Targeting CRHR1 has been challenging; drug development has been unsuccessful despite compelling preclinical data, CNS activation with imaging and some clinical studies showing beneficial effects. A molecular imaging agent has not been forthcoming despite much focused effort by competent investigators. Capturing a better understanding of CRH/CRHR1 involves more study at a molecular and systems level. Knowledge of how the stress system interfaces with the vulnerability, genesis and continuance of clinical disorders requires more work. As highlighted commonalities with neurochemical and circuit activation/deactivation patterns do not form a one to one map with clinical disorders and this would not make physiologic “sense.” The commonality of stress dyshomeostasis is multi-dimensional and finding differences not similarities seems key to achieving increased specificity. Additional targets molecular relevant to stress are being tested in clinical populations or are near clinical trials. The Table 1 provides a partial list of targets that represent current research directions.

Conclusions—These are evolutionary conserved systems, ancient molecules with major roles in internal homeostasis and environmental adaptability of organisms. As the central nervous system became more complex these molecules diverged and developed greater influence on behavioral adaptations to changing environmental conditions. Primate evolution found distinct advantage in a stress system as a top down behavioral and somatic control “governor.” CRH/CRHR1 expression progressed from subcortical to cortical areas with broader and shifting control loci regulating and integrating higher cognitive and executive functions.

CRH has shown to be fundamental in the regulation of stress which has allowed science to build from our knowledge of rodents to NHP and now to clinical disease. Most studied are psychiatric disorders, undoubtedly the most complex and least understood human conditions. These most often represent a constellation of common symptoms and signs poorly discriminating one from another. As noted by Kapur et al. they discuss “Approximate Replications,” in imaging and molecular medicine; there are seldom problems differentiating healthy controls from patients exhibiting clear manifestations of disease. What is critical is delineating often contradictory findings in more or less homogenous patient populations. A cardinal issue are ill-defined variables influencing both directionality and magnitude of findings making clinical application of studies such as fMRI and PET unreliable. Despite

finding significance using statistical measures ($p < 0.05$) the effect sizes of these results are limited and clinical relevance to patient therapy and management even less [134].

Applying the burgeoning knowledge of the stress system to imaging research is nascent; the concept of "Approximate replications" is relevant but more challenging is defining what "normal" stress is? Susceptibility to stressful environments or experiments draws back to state or trait characteristics which are inherently difficult to quantify. Neurochemical, metabolic and cognitive research employ a variety of statistical methods to determine normative values and relevant changes from the mean. Stress is a largely an unknown variable. The benefit of considering the stress milieu in imaging is becoming more recognized. Integrated assessment of stress activation can be determined by maneuvers such as neuroendocrine testing, assessment of cortisol deposition in hair and determination of specific alleles for stress modifying genes [133, 37]. However how this would improve specificity is unknown.

The authors have shown stress system activation, both acute and chronic will induce changes in receptor expression, neuronal integrity, network connectivity and permanent or semi-permanent changes in CNS "hard wiring." All can add variability in research findings and when added to underpowered statistics due to small group and effect size, differences in study design and methodology contradictory results occur despite $P < 0.05$ and chances for future replication can be < 50%. These factors all contribute to failure to reach "gold-standards" for clinical applications [134].

Achieving "Stratified Medicine" will be a challenging task that has largely failed over the last 50 years. Imaging technologies enhance our ability to segregate and characterize individuals; the specificity of these effects is dependent on the investigators accurately identifying variables important to the answering focused questions. Many factors will require refinement and improved precision; increasing effect sizes to enable clinical application and importantly the ability to distinguish subtle but often critical signs in a constellation of otherwise similar symptoms. Understanding and applying the knowledge of how stress can influence disease at a molecular and systems level will be critical to adopting adjunct biological markers to clinical practice [130]. It is unlikely that accounting for these variables alone will achieve the desired outcomes. Incremental improvements are all we may reasonably expect.

What is normal? Stress permeates everyone's life. Its impact on our brain, our behavior and our psyche can be profound. With the remarkable sensitivity of evolving imaging technologies seemingly inconsequential stressors, such as a new or novel situation may have discernable neurochemical effects when focused with the right tools. The cumulative experience of behavioral, chemical or other environmental exposures maybe become imbedded in the fabric of our brain. Current clinical assessment of disease can be too blunt an instrument in some cases to predict subtle (or not so subtle) changes in structure, connectivity or neurochemistry. Addressing uncertainty is a key element in any scientific discipline, establishing the range of "normal" and disease requires empiric testing. Defining the many variables as have been discussed may be the initial step in quantifying and

directing future investigations. Advancing imaging technologies may offer another metric to assess these effects.

How do you define a “normal” emotional responses if it can leave a permanent, indelible mark and how do you differentiate those findings from disease? The answers await refinement of our understanding of stress and its consequences as we move towards stratified medical diagnoses and their biologic applications.

Abbreviations

CNS	Central nervous system
CRH	corticotropin releasing hormone
CRHR1	corticotropin releasing hormone receptor type 1
irCRH	immune-reactive CRH
PET	positron emission tomography
SPECT	single photon tomography
fMRI	functional magnetic resonance imaging
DTI	diffusion tensor imaging
pMRS	proton magnetic resonance spectroscopy
PFC	prefrontal cortex
dlPFC	dorsolateral prefrontal cortex
dmPFC	dorsomedial prefrontal cortex
aCC	anterior cingulate cortex
EA	extended amygdala
VTA	ventral tegmental area
SNS	sympathetic nervous system
DR	dorsal raphé nuclei
LC	locus coeruleus
BBB	blood brain barrier
HPA	hypothalamic pituitary adrenal axis
AVP	vasopressin
ACTH	adrenocorticotropin
C	cortisol
GCr	glucocorticoid receptors
BNST	bed nucleus of the stria terminals
NAc	nucleus accumbens

MDD	major depressive disorder
GAD	generalized anxiety disorder
PTSD	post-traumatic stress disorder
NHP	non-human primate
SS	stress sensitive
SR	stress resistant
DA	dopamine
5HT	serotonin
ERC	entorhinal cortex
5HTT	serotonin transporter
GABA	gamma-aminobutyric acid
BOLD	blood oxygenation level dependent
sCC	subgenual cingulate cortex
MD	maternal deprivation
GCr	glucocorticoid receptor
RAC	raclopride
GC	glucocorticoid
GLU	glutamate
mGluR5	metabotropic GLU receptor subtype 5
ETOH	alcohol
DAT	dopamine transporter
NET	norepinephrine transporter
BP	binding potential
SP	substance P
NK-1	Neurokinin1
O/H	Orexin/Hypocertin
END	beta-endorphin
CFN	carfentanil
TSST	Tier Social Stress Test
FDG	fluoro-2-deoxy-D-glucose
FA	fractional anisotropy
BMI	body mass index

IRPCOS	insulin resistant polycystic ovary syndrome
IBS	irritable bowel syndrome
rCMglu	regional cerebral glucose metabolic rate

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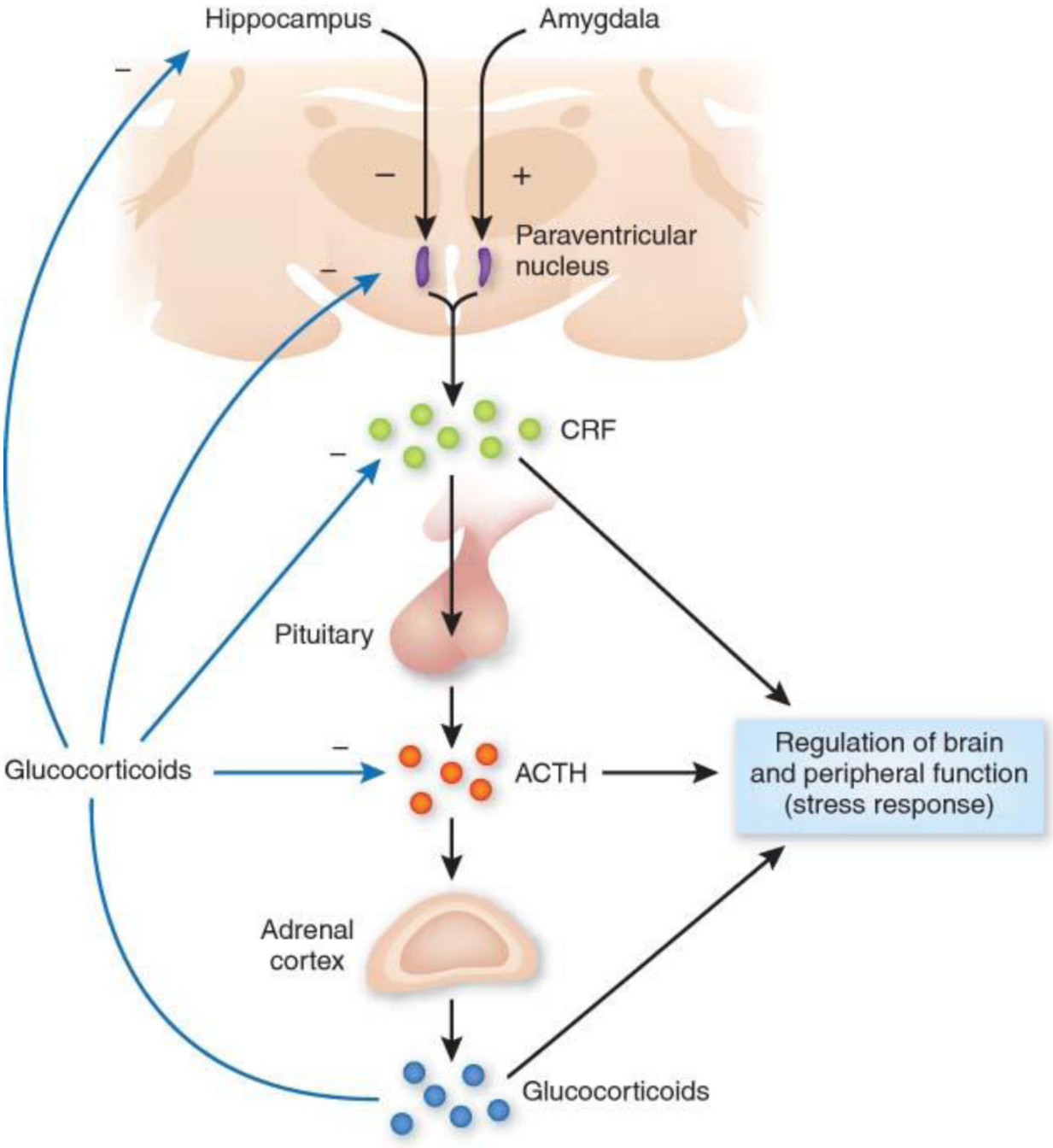


Figure 1.

Hypothalamic pituitary adrenal axis regulation of glucocorticoid/cortisol secretion in response to an acute stressor; this demonstrates multiple levels of feedback inhibition to the pituitary, hypothalamus and limbic structures critical for the autonomic and behavioral responses to stress.

Nature Neuroscience 12, 241 – 243 (2009) 16 March 2009 doi:10.1038/nn0309-241

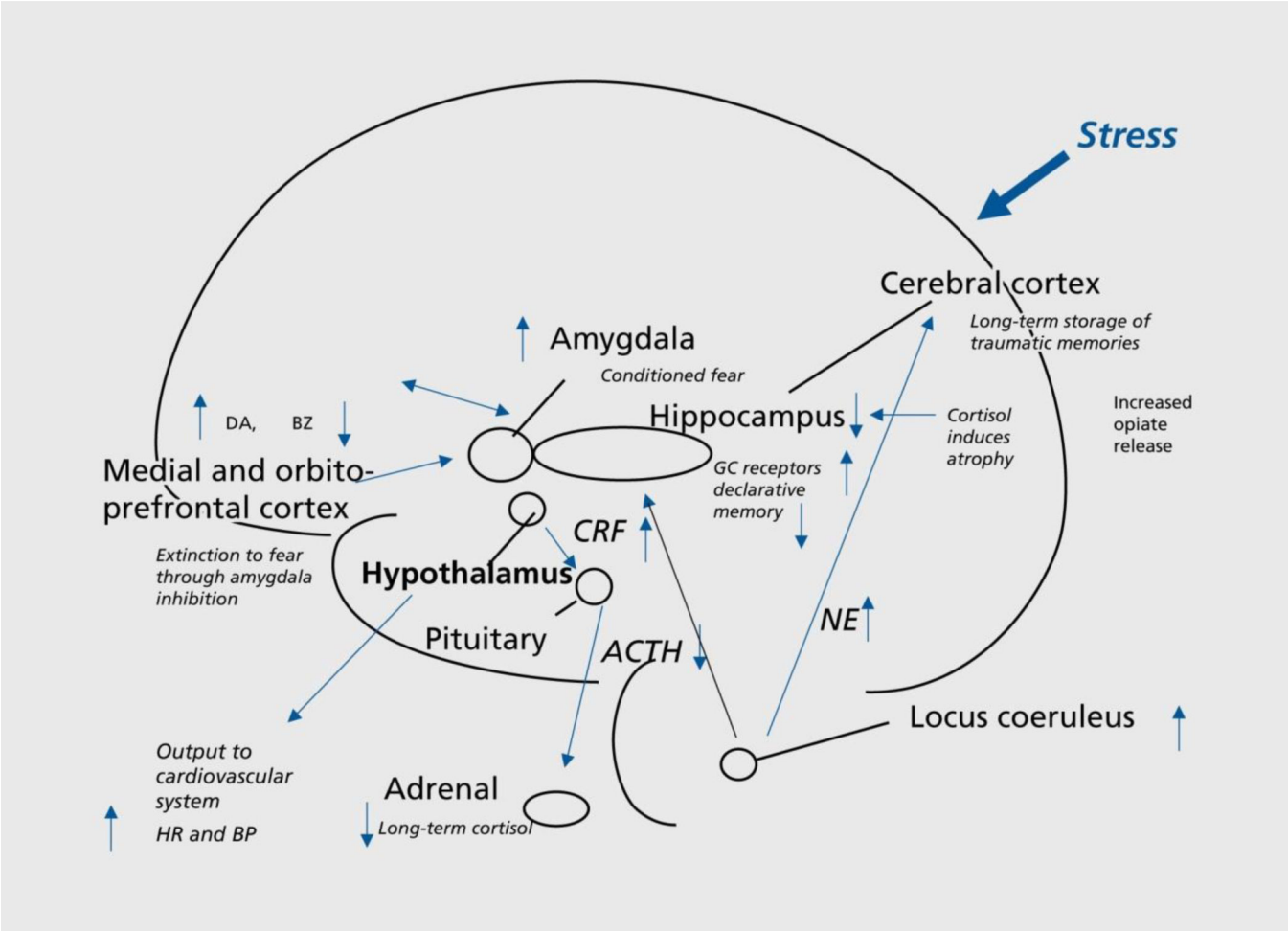
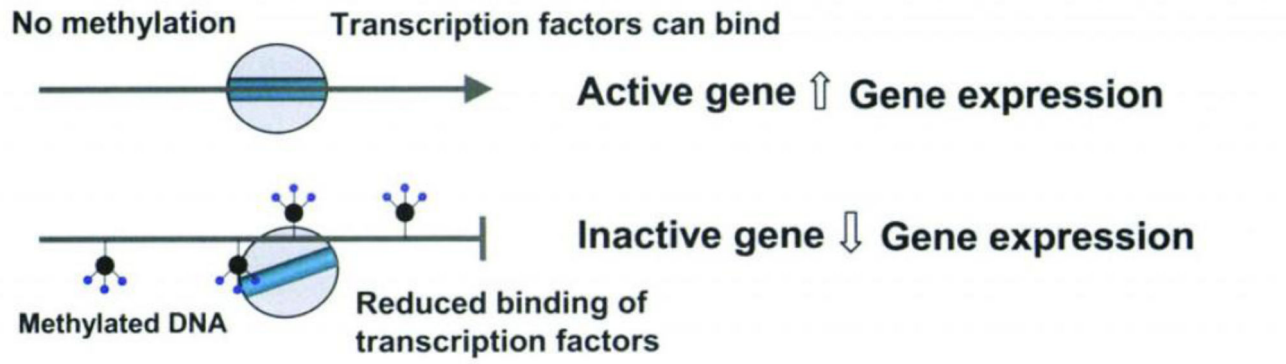
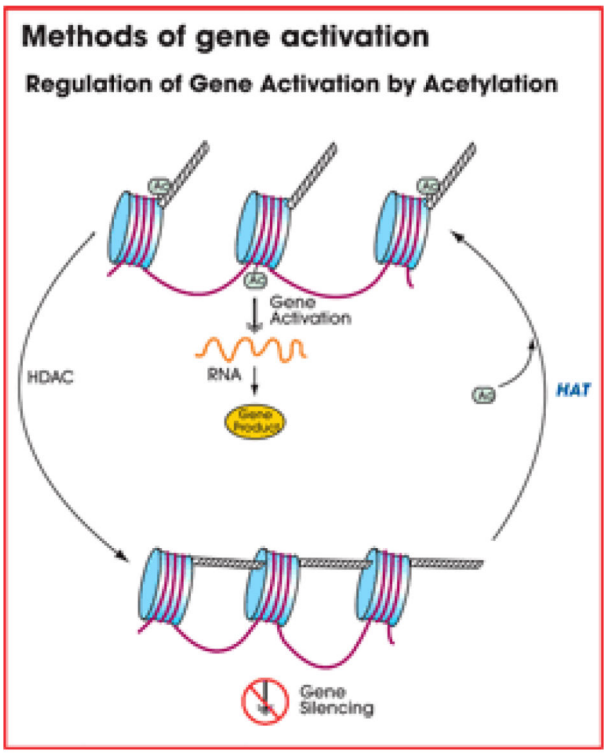


Figure 2.

Schematic diagram shows the interface and complexity of multiple structures and functional neurochemical interactions during the chronic adaptation to stress. Normal responsivity of the HPA is lost, chronic activation of cortisol damages hippocampal memory consolidation, decreases prefrontal cortex suppression of a hyper-responsive amygdala; elevated cortisol and excitatory neurotransmitters (i.e. glutamate) can cause neuronal and white matter injury and atrophy. Other neurotransmitters and neuronal networks are subsequently affected. DA-dopamine, BZ – GABA and GABA agonists, GC – glucocorticoid/cortisol receptors, NE – norepinephrine, ACTH – adrenocorticotrophin, CRF - corticotropin releasing hormone, opiate – endorphin and related opioid peptides.



Methylation of histones will block gene activation and transcription



Histone acetylation is permissive for gene transcription and gene activation
http://www.psychweekly.com/aspx/article/articledetail.aspx?articleid=1143

Figure 3. EPIGENETIC MECHANISMS

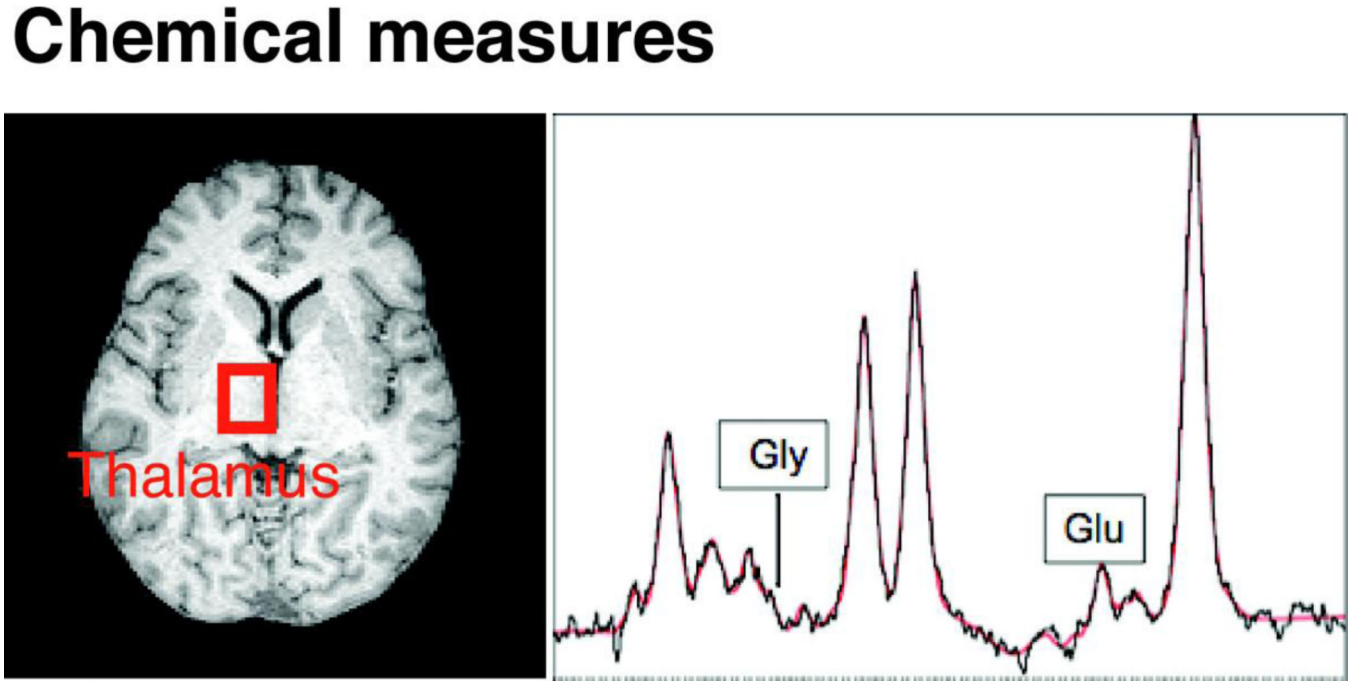


Figure 4.

Proton Magnetic Resonance Spectroscopy region of interest spectra over the thalamus for recording glycine and glutamate/glutamine peaks for determination of the neurotransmitters and metabolites. The relative concentration of neurotransmitters can be quantified with this technique.

Borsook *et al. Molecular Pain* 2007 **3**:25 doi:10.1186/1744-8069-3-25

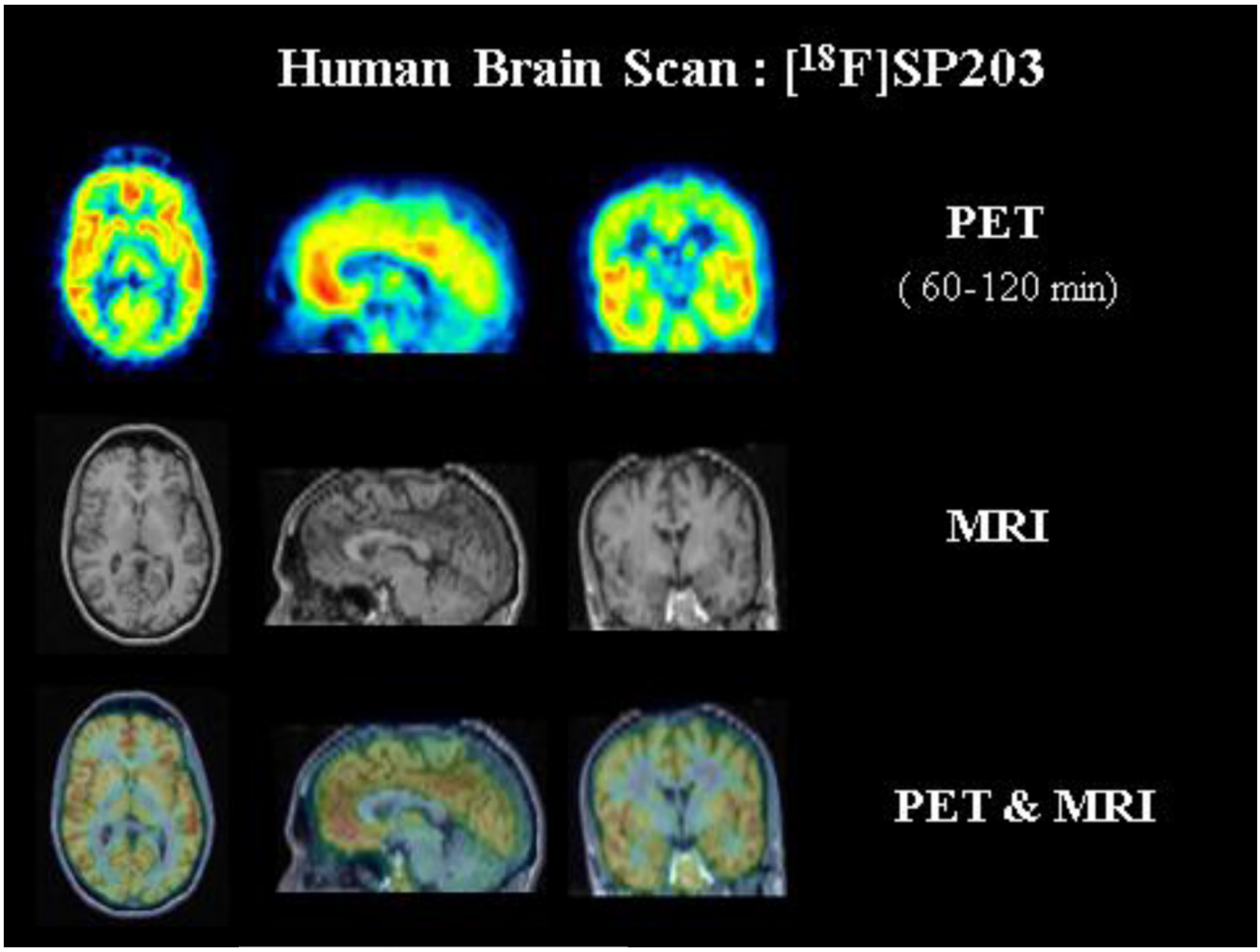


Figure 5.

PET and MRI images of mGLUr5 PET ligand [^{18}F] SP203, showing raw and MRI fused receptor distribution in a human volunteer.

Courtesy of Dr Robert Innis, National Institutes of Health, National Institute of Mental Health

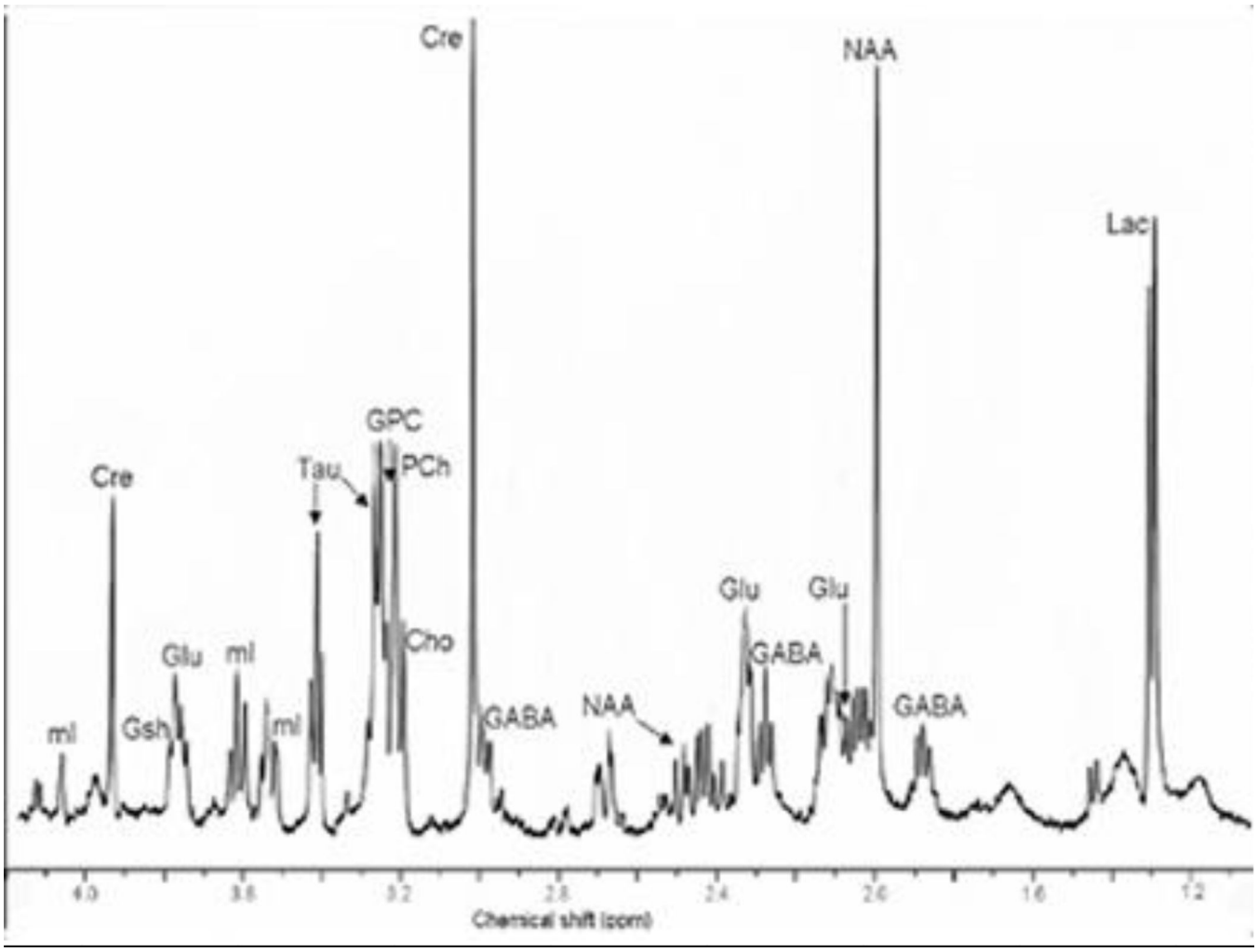


Figure 6.

Representative Proton Magnetic Resonance Spectroscopy (pMRS) signal from brain showing multiple peaks for CNS metabolic markers and neurotransmitters. Cho-choline, Cr-creatine, NAA- N-acetyl aspartate, Lac- lactate, GABA - *gamma*-aminobutyric acid, Glu-glutamine/glutamate, ml - myoinositol, PCh - phosphocholine, GPC - glycerophosphocholine Tau - taurine, Gsh - glutathione

http://pubs.niaaa.nih.gov/publications/arh341/99-105.htm

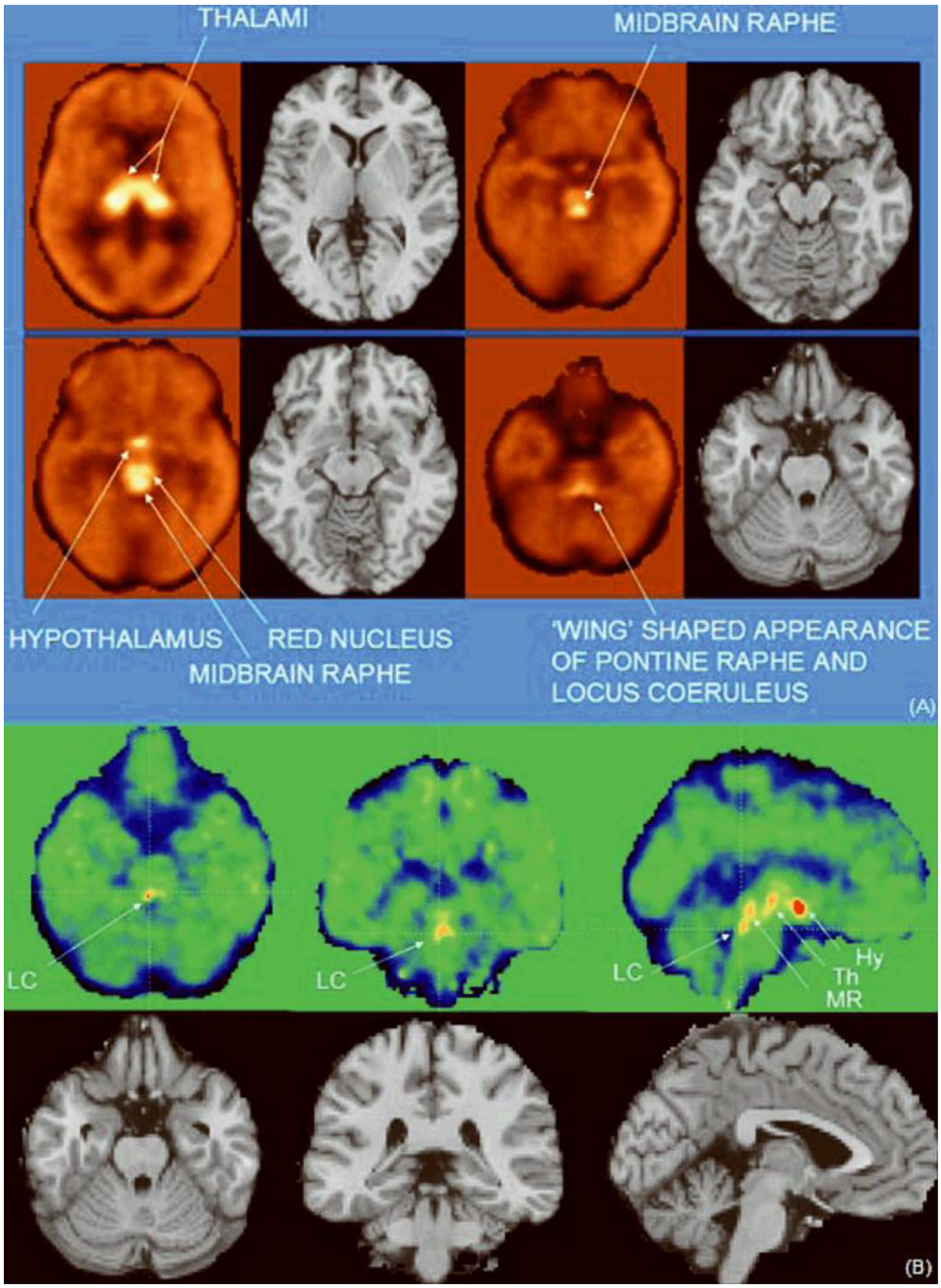


Figure 7.
Distribution of [^{11}C]methylreboxetine for the norepinephrine transporter (NET) in thalamus, hypothalamus and brainstem nuclei.

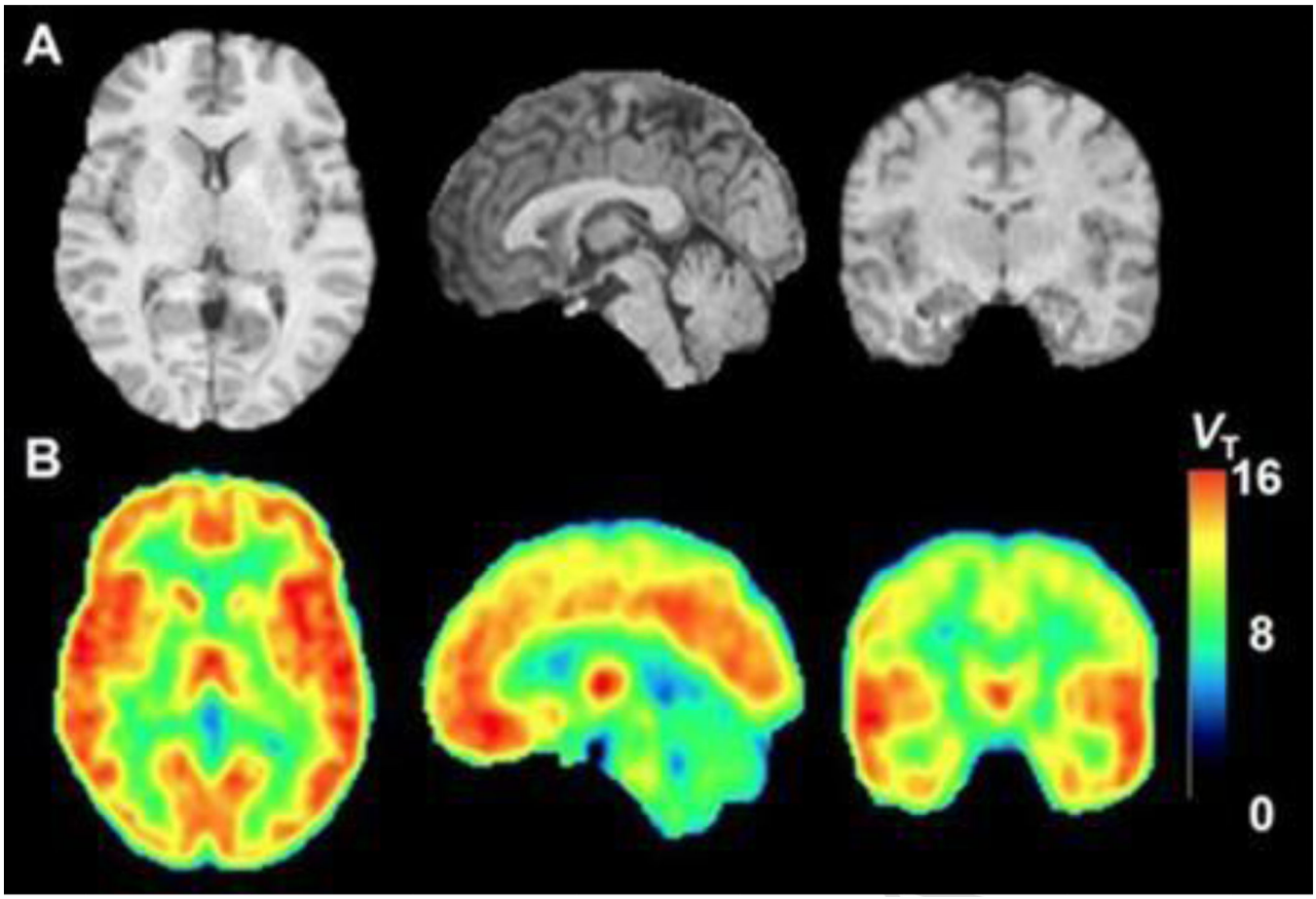


Figure 8.

Nociceptin PET Scan:

This shows a first generation radiotracer for the neuropeptide Substance P/Nociceptin; the neuropeptide shows wide expression throughout critical stress regulating cortical areas i.e. prefrontal, cingulate, frontal, and temporal lobes; it is also seen in subcortical regions of the extended amygdala, entorhinal cortex and portions of the striatum.

http://www.psychologytoday.com/blog/the-athletes-way/201401/scientists-discover-elixir-stress-called-nociceptin

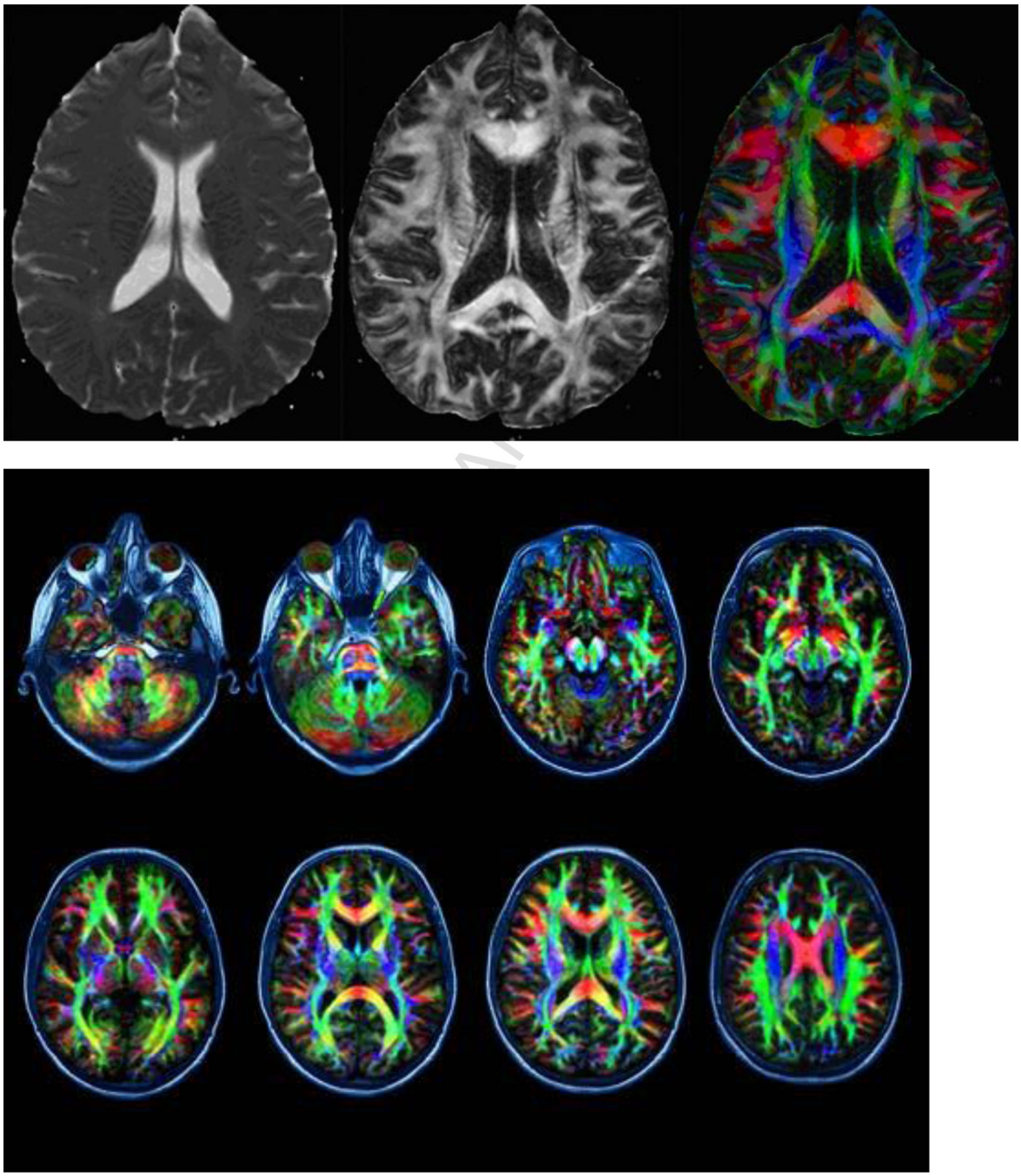


Figure 9. Diffusion Tensor Imaging

Diffusion Tensor Imaging builds on MRI technology to measure the integrity of the brain's white matter. White-matter communicates between brain regions and the spinal cord. White matter damage can have serious, long-term consequences; pathologic changes are often seen in neurodegenerative disorders and psychiatric conditions where chronic stress is often contributory. In these images axons are colored according to orientation; fibers running between the front and back are blue, those between right and left are red, and those running

between the brain's interior and exterior are green. Disruption in these tracts can be quantitated, are reproducible and validated in normal and disease states.
http://www.pbs.org/wgbh/nova/sciencenow/0306/02-diag-03.html
http://virtualneuro.net/neuroblog/wp-content/uploads/2010/06/temp48.jpg

Table 1

Possible future targets for detecting stress response with current imaging technologies.

FUTURE TARGETS
Glucocorticoid receptors
CRHR1 Antagonists Agonists
New radiotracers Glutamate GABA Norepinephrine Substance P/Neurokinin1 Nociceptin/Orphanin FQ Orexin/Hypocertin
Second Messenger Targets Tyrosine and protein kinase inhibitors Intracellular small molecules Membrane receptor based
Genetic/Epigenetic markers Intracellular/intranuclear small molecules
Neuroreceptor PET, structural, cognitive fMRI,MRS Associated activation markers Deactivation markers MRI receptor specific contrast agents