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Assessing the Neuroendocrine Stress Response in the Functional Neuroimaging Context

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Abstract

Neural regulation of stress responses, and the feedback of stress hormones to the brain, reflect complex brain-body interactions that may underlie the effects of psychological stress on health. Elucidating the brain circuitry involved in the cortical control of limbic-hypothalamic-pituitary-adrenal axis, and the cortical “targets” of cortisol that in turn modulates brain function, requires careful assessment of glucocorticoid hormones, in the context of the neuroimaging paradigms. Here we discuss approaches for assessment of endocrine function in the context of neuroimaging, including methods of blood and saliva specimen collection, and methods for drug/hormone administration. We also briefly discuss important temporal considerations, including appropriate timing of sample collections for hormones with different time-courses of activation (e.g. ACTH vs. cortisol), the pharmacokinetics of both endogenous hormones and administered agents, and circadian considerations. These are crucial to experimental designs of rhythmic hormonal systems and multiple feedback loops. We briefly address psychological/behavioral ‘activation’ paradigms used for inducing endogenous LHPA axis responses within or in proximity to scanner, as well as strategies for administration of exogenous hormones or secretagogues. Finally, we discuss some of the analyses issues in terms of hormone responses (e.g. response and area under curve, diurnal variability) and strategies for linking measured levels of peripheral humoral factor to brain activity (e.g. hormone responses as between subject regressors of BOLD activations, hormone levels as within subject regressors in analyses of covariance of brain activity over time, etc.).

Introduction

Homeostatic hormonal systems like the limbic-hypothalamic-pituitary-adrenal (LHPA) axis ultimately are under neural control, mediated largely by neurosecretory inputs to the anterior pituitary. The activity of these systems is complex and can be thought as containing two principal components that can interact and influence each other: 1) baseline activity and 2) responses to specific events/challenges. The baseline activity of classical pituitary “neuroendocrine axes” are generally thought of as being under neural control of circadian (daily) rhythms, largely mediated by the suprachiasmatic nucleus (Bao et al., 2008; Czeisler and Klerman, 1999; Dai et al., 1998). They involve cascades of “releasing” and “inhibiting” hormones from hypothalamic neurons, which are secreted into the hypothalamo-hypophyseal (portal) circulation to bind receptors in specialized secretory cell types in the pituitary. These

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pituitary cells then secrete “tropic” peptide hormones, which travel through the general circulation to the peripheral target organs to stimulate secretion of the hormonal “end products” (for review see (Tsigos and Chrousos, 2002)). The circadian hormone rhythms themselves are comprised of more rapid pulsatile secretions (often in approximately hourly pulses) of the end product, known as “ultradian” rhythms. (Lightman et al., 2008). For example, anterior pituitary systems regulate function of thyroid gland (thyroid stimulating hormone (TSH) regulating thyroxine), gonads (gonadotropins regulating sex steroids), and liver (growth hormone (GH) regulating production of insulin-like growth factor I (IGF-I)). (Tsigos and Chrousos, 2002) The posterior pituitary in turn secretes melatonin, as well as oxytocin, and vasopressin, which have become increasingly recognized to influence physiology and behavior (Carter, 1998). Each of these classical neuroendocrine cascades have been elegantly mapped over the past 30 years within animal models (e.g. rodent, sheep, and monkey) using combinations of lesion, stimulation, and pharmacological approaches. Baseline function of these systems is under circadian (and monthly or “circalunar”) control, and it can be influenced by environmental and psychological factors, suggesting a mechanism for psychological modulation of physiological systems (Bao et al., 2008; Edwards et al., 2001; Hallonquist et al., 1986)

In the case of the LHPA axis, the hormonal cascade consists of corticotrophin releasing hormone (CRH) (and in some instances, vasopressin) secretion from parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus, which binds to receptors on adrenocorticotrophic cells of the anterior pituitary, stimulating release of adrenocorticotrophic hormone (ACTH). ACTH in turn binds to receptors in the adrenal cortex and stimulates production of the “end product” of this cascade (cortisol in humans and corticosterone in rodents) (for review see (Bao et al., 2008; de Kloet et al., 2005). The LHPA axis has a pronounced circadian rhythm (in humans, peak plasma cortisol levels in the morning and lowest cortisol levels at night) comprised of ultradian rhythms of approximately hourly secretions of cortisol from the adrenal gland. In addition to circadian rhythm, the LHPA axis, can be directly activated by psychological as well as physiological stressors (e.g. pain, tissue damage, glucose levels), thus providing mechanisms for integrated control of physiological functioning from the brain to meet changing environmental (including social) demands.

The neurocircuitry underlying the psychological “drivers” of the LHPA axis have been mapped in animal models (restraint, predator exposure, etc.), and include subcortical structures such as the amygdala and the bed nucleus of the stria terminalis (BNST) which have multisynaptic projections (via proximal GABA interneurons) to the CRH-secreting PVN neurons (Herman et al., 2005). However the “upstream” cortical control of these processes is less well understood. In humans, the psychological factors that most reliably activate the HPA axis are novelty, social evaluative threat, and perceived controllability (Abelson et al., 2005; Dickerson and Kemeny, 2004). These are complex factors, and recent evidence suggest, for example, that perceived controllability can even influence the effects of a direct pharmacological secretagogue (Abelson et al., 2008; Abelson et al., 2005). This suggests that complex cortico-limbic circuitry is involved in the control of LHPA axis response, which unlikely to be elucidated by the use of rodent models exclusively.

Corticosteroids have a multitude of biological effects in almost every tissue in the body. Peripheral effects include regulation of metabolism and glucose deposition, including stimulation of lipolysis, inhibition of glucose uptake into muscle and fat, and stimulation of gluconeogenesis (and hence the name “gluco”-corticoid). There are also important effects on immune function (primarily immunosuppressant, and hence the widespread pharmacological use of “steroidal immunosuppressants”), as well as diverse effects on cardiovascular tone and development, among others (for review see (Newton, 2000). The central nervous system/brain effects of glucocorticoids have been the focus of study in the past 2 decades in animals (e.g.

rodents and sheep) and more recently in humans. Initial evidence suggests that cortisol modulates cognitive and emotional processing, social appraisal, and decision making (Erickson et al., 2003). Thus there appears to be “two-way traffic” between the LHPA axis and brain activity, (for review see (de Kloet et al., 2005)). Glucocorticoids are steroids that readily cross the blood-brain barrier and directly bind to intracellular receptors across the brain, that potentially mediate the effects of cortisol on cognition, emotion, and decision making. Understanding the sites and potential mechanisms of action of hormone on the brain will be furthered by administration of exogenous hormone during neuroimaging, probing for specific neurocircuitry.

The cellular effects of corticosteroids are mediated through binding to intracellular receptors of the “nuclear receptor” superfamily. These receptors are transcription factors which reside in the unbound state in the cytoplasm, in association with specific chaperone proteins (e.g. heat shock proteins and FKBP5 (Grad and Picard, 2007; Heitzer et al., 2007)). Glucocorticoid binding leads to dimerization and translocation of the receptors into the cell nucleus, where they are active transcription factors that operate genomically to regulate gene expression through interaction with specific promoter response elements such as the “glucocorticoid response element” (or GRE), found in many glucocorticoid-regulated genes (Newton, 2000). There are two known receptor subtypes of corticosteroid receptors, known as the Type II, or glucocorticoid receptor (GR), and Type I, or mineralocorticoid receptor (MR) so called because it is also involved in electrolyte balance in the kidney (for review see (De Kloet and Derijk, 2004; de Kloet et al., 2005)). The MR is a high affinity, low capacity receptor (which actually has higher affinity for glucocorticoids than the GR), while the GR is a relatively lower affinity, higher capacity receptor. In rodents, the GR has highest levels of expression in the hippocampus, PVN hypothalamus, and pituitary, but is also distributed throughout multiple cortical regions, including frontal cortex; whereas the MR expression is largely confined to the hippocampus (Jacobson and Sapolsky, 1991; McEwen et al., 1986; Reul and de Kloet, 1985). Interestingly, in human and nonhuman primates, both GR and MR are widely expressed throughout the neocortex, as well as PVN and hippocampus (Sanchez et al., 2000; Xing et al., 2004). At physiological glucocorticoid concentrations, the high affinity MRs are usually tonically occupied and active in the nucleus. Thus while MR do not appear to directly mediate the effects of “stress-levels” of glucocorticoids in the brain, they do likely maintain neuronal integrity and a stable excitatory tone, in particular in the hippocampus, and may be involved in the tonic inhibition that the hippocampus exerts (via inhibitory interneurons) on PVN CRH-secreting neurons (Joels, 2008; Joels et al., 2004). The lower affinity, high capacity GRs are occupied mainly during ultradian peaks (stress-levels) of cortisol. Therefore the GR has been thought to involved in cortisol inhibitory feedback, as well as “stress-responsive” cortisol effects on brain activity, such as in emotional processing and memory. However, the specific mechanism by which glucocorticoids exert these effects on specific neurons, and how these lead to changes in functional neurocircuitry, is yet to be elucidated. One area of glucocorticoid effects on the brain that has been studied is glucocorticoid negative feedback, the process by which circulating glucocorticoids regulate their own levels, through binding brain receptors. Glucocorticoids decrease the CRH secretion from the PVN hypothalamus, and effects that appears to mediated primarily by GR, and thus dampens LHPA axis activation (Herman et al., 2005). Work over the past two decades in rodents has also demonstrated important roles of hippocampus and medial frontal cortex as sites of glucocorticoid feedback actions (Diorio et al., 1993; Furay et al., 2008; Sullivan and Gratton, 2002), reviewed in (Herman et al., 2005). In addition to “negative feedback”, glucocorticoids are also able to “sensitize” aspects of subsequent stress responses through “feed-forward” mechanisms, for example, by elevating levels of “extra-hypothalamic” CRH in limbic structures such as the amygdala. Repeated stressors can lead to further elevations in amygdala CRH, an effect blocked by a glucocorticoid receptor antagonist mifepristone (Cook, 2002, 2004). Glucocorticoids can also lead to changes in gene expression of stress mediators in limbic regions, such as up-regulation of CRH gene

expression in the amygdala and BNST (Schulkin et al., 2005) that may lead to longer term “feed-forward” or sensitization of stress responsiveness.

Given the role of GR and MR as transcription factors, their effects on brain and emotional processing have traditionally been conceptualized in terms of genomic effects. However, “fast” effects of glucocorticoids in neurons and in brain that are too rapid to be explained by genomic effects have been demonstrated for decades (Dallman, 2005). Recent work suggests some of these effects involve rapid glucocorticoid suppression of miniature excitatory postsynaptic currents (mEPSC) in magnocellular neurons of the PVN, and are thought to be mediated by binding to less well characterized, MR-like membrane bound sites (de Kloet et al., 2008; Joels et al., 2008). The mechanism by which these effects are translated to parvocellular PVN neurons that secrete CRH may be complex, and appear to be G-protein dependent and involve endocannabinoid system (Di et al., 2003; Tasker et al., 2006). Several other rapid effects of glucocorticoids on neurons have been reported, including effects on membrane excitability (Lupien and McEwen, 1997), potassium channels in PVN (Zaki and Barrett-Jolley, 2002), NMDA receptor calcium signaling in hippocampus (Takahashi et al., 2002) (Venero et al., 1993) and raphe nucleus neurons (Avanzino et al., 1983), suggesting rapid, dynamic modulation of brain activity by cortisol may be possible.

Repeated activation of the HPA system either by repeated stressor exposure or by “mis-appraisal” of environmental cues, or failure to appropriately terminate LHPA responses, has been suggested to have a cumulative effect on the body (i.e. “allostatic load”) and may lead to increased risk for negative health outcomes, such as diabetes, hypertension, cancer, and cardiovascular disease (de Kloet et al., 2005; McEwen, 2003, 2006). This suggests that better understanding of complex cortical control of LHPA axis in humans is urgently needed. *In vivo* functional neuroimaging can help to elucidate the potentially complex cortical circuitry which provides the ‘top-down’ regulation of the LHPA axis. Neuroimaging research aimed at tracking peripheral hormones during ‘activation’ paradigms while also manipulating specific cognitive factors (Abelson et al., 2008) may help to delineate the specific neural circuits involved. Recently, a number of studies have taken advantage of these strategies of linking brain activity with peripheral levels of plasma ACTH (Liberzon et al., 2007; Ottowitz et al., 2004), King et al. this issue), plasma cortisol (Bonne et al., 2003; Drevets et al., 2002; Liberzon et al., 2007), salivary cortisol (Pruessner et al., 2008; Urry et al., 2006; Wang et al., 2005), and plasma cortisol sensitivity/eosinophil activity (Rosenkranz et al., 2005). A small number of studies have examined brain activity in specific psychological paradigms such as viewing emotional faces and memory tasks after hormone administration, including cortisol ((de Quervain et al., 2003) Sudheimer, Ho, Garfinkel, & Liberzon, unpublished data). Further work is needed to elucidate the cortical neurocircuitry underlying psychological influences on control of neuroendocrine function, and the cognitive and affective effects of these hormones. In the following sections we will describe some basic methods and considerations for experimental design and analytical strategies for this kind of work.

Materials and methods used in assessment of LHPA axis in neuroimaging

At their most basic levels, study designs for elucidating the neurocircuitry involved in psychological activation of hormone systems involve measurements of hormone concentrations or activity at baseline and after “activation”, and relating these measures to brain activity. The choice of specimen collection schedule and the nature of behavioral activation or stress induction paradigm will depend upon the specific time-course and characteristics of the particular hormone cascade and the specific brain function under study. In the following paragraphs we briefly describe methods for collection of blood and saliva specimens within the scanner itself and/or proximal to scanning, as well as strategies for and issues involved in administration of exogenous hormone/pharmacological agents (e.g. agonists

and secretagogues) via oral and intravenous methods. Finally, we discuss some of the analytical issues in terms of hormone responses (e.g. response and area under curve, diurnal variability) and strategies for linking peripheral humoral factor to brain activity (e.g. between subject regressors).

Methods and procedures for blood and saliva specimen collection

Collection of biological specimens (e.g. blood and/or saliva) for measures of circulating hormones during the scanning procedure requires access to the participant, thus SPECT and PET provide easier access than fMRI, in which the participant is in a tube and the scanner is usually in a sealed room. This advantage however is clearly mitigated by general availability and better temporal resolution of fMRI. With respect to plasma specimen collection, PET and SPECT studies involve intravenous access (to deliver radiotracer), thus the subject will have already consented to the placement of indwelling catheter for the purpose of the study. However the majority of fMRI studies don't involve IV catheters, thus plasma specimen collection will require additional IRB considerations, specific consent, and staff trained and certified in placement of IV catheters. Indwelling catheters with tips compatible for the MR environment are widely available (e.g. as used for injection of contrast agents) and can also be used to sample blood during fMRI, but may require either a long line to allow sampling in the control room (which could present technical difficulties), or that a technician enter the MR room and pull samples at desired times.

Plasma—Circulating blood is the compartment most proximal to secretion of LHPA axis neuroendocrine factors (e.g. CRH, ACTH, cortisol) and potential psychological 'stress' effects. Collection of plasma may also allow for measurement of many additional peptide or protein hormones (e.g. oxytocin, opioids), catecholamines, cytokines, etc. The half life of hormones in general, and of peptides in particular, is relatively very short in plasma as they are readily inactivated or broken down by specific peptidases and other enzymes, thus the specimens are usually handled on ice (protease inhibitor "cocktails" can be added to minimize degradation), and processed within 30 minutes or so. It should be noted that a large proportion of steroid hormones (e.g. cortisol and sex steroids) in blood are bound by circulating serum albumin and specific binding globulins (e.g. cortisol binding globulin (CBG) and as little as 3–5% of steroids exist in the bio-available unbound or "free" form (Coolens et al., 1987). Commercial kits and services are widely available for the assay of neuroendocrine hormones (e.g. CRH, ACTH, cortisol), and other blood factors (e.g. cytokines).

Saliva—Measurement of cortisol in saliva provides the opportunity to collect samples in a relatively low stress manner and without medical personnel. A common method for obtaining saliva specimens is to have participants chew a cotton roll, which becomes saturated by saliva (a commercially available system, the "Salivette" (Sarstedt, Inc. Mannheim, Germany) contains a roll of dental cotton in a inner plastic tube with a frit at the bottom, which can be centrifuged to easily collect saliva (ca. 0.5 – 1.5 ml) in an outer tube.) Saliva samples can be stored at room temperature for several days without significant breakdown of cortisol because it has less hormone degrading enzymes than plasma. Subjects can also manually "express" saliva (spit) directly into a tube. Collection of saliva is less invasive than venipuncture; however, collection of saliva during a scanning session requires access to the participants mouth, and also is complicated by potential head movements made by chewing collection cotton or spitting. There have been attempts to address some of these issues in recent reports (Dedovic et al., 2005; Pruessner et al., 2008) by moving a subject only partially out of the scanner, just enough to access the mouth, and having a researcher insert a cotton roll into the subject's mouth using a gloved hand; the subject then keeps the roll in the mouth without chewing, allowing it to saturate with saliva passively for about 2 minutes, and then expresses it out of the mouth with the tongue, and the researcher retrieves it again with a gloved hand.

While this can be done minimizing head movements, it still requires the subject to interrupt the scanning session and interact with researcher multiple times. .

Saliva contains measureable levels of several steroid hormones, including cortisol, as well as DHEA-sulphate, estrogen, progesterone, and testosterone (Atkinson et al., 2008). Steroid hormones enter saliva by diffusion of the free steroid through the acinar cells of the salivary glands, with rapid equilibration between saliva and free cortisol in serum. Salivary cortisol exists primarily in a free form, with only <15% bound to protein, and levels are highly correlated to serum free cortisol levels, and empirical work suggests salivary measures may actually have an advantage in terms of convenient measurement of free steroid hormones (Gozansky et al., 2005), and is now very widely utilized as a marker of plasma cortisol levels. While recent data also finds some limitations in the ability of saliva cortisol to accurately assess plasma levels (i.e. that oral contraceptives, estrogen levels, and levels of CBG may confound the correlation of saliva and plasma cortisol levels (Hellhammer et al., 2009)); overall, saliva cortisol appears to be a highly valuable marker of HPA axis activity. In addition to steroid hormones, some cytokines (TNF- α , IL-1 β , sIL-2) (Dugue, 1996) have also been successfully measured in saliva, and recently measurement of some small peptides in saliva (e.g. oxytocin) has also been reported (Carter et al., 2007; Horvat-Gordon et al., 2005).

Studying LHPA axis function or stress responsiveness

The first important question in designing a study of this kind is the choice of LHPA axis hormones to measure. CRH release from the PVN into the portal circulation is the neurosecretory event that begins the cascade, thus measurement of peripheral CRH responses would appear to be the best choice for relating to brain activity. However, there are technical limitations to measurement of peripheral CRH in the plasma. In humans, except during pregnancy (during which placenta CRH is secreted (Florio et al., 2007)), peripheral CRH concentrations are very low (<10 pg/ml), and appear to be primarily from extra-hypothalamic sources (Sasaki et al., 1987) and do not reflect local concentrations within the portal circulation seen by andenocorticotropes in the anterior pituitary. Plasma CRH responses to psychological stress are often not measureable, and are not correlated with subsequent ACTH or cortisol levels; thus severely limiting the usefulness of plasma CRH as a probe for brain activity. In contrast, both ACTH and cortisol have robust responses that are easily measured in plasma (and cortisol, in saliva). In humans, as in rodents, increases in the plasma concentration of ACTH are measurable as early as 1–5 minutes after the stressor, whereas stimulated plasma (and saliva) cortisol levels have a considerably longer latency (owing to the time for ACTH to increase adrenal cortical steroidogenesis) with increases measurable at 10 min but not peaking until ca. 20–30 min, and recovery in 45–60 min (Dickerson and Kemeny, 2004; Kirschbaum et al., 1993a; Kudielka et al., 2009; Kudielka et al., 1998). Thus generally speaking, endocrine events are much longer-lasting compared to the neural events which “drive” them. Thus studies that use cortisol to examine neural correlates of stress-activation of HPA axis must make allowance for the relatively long latency of response, and may involve measurement of brain activity at the time of the stressor and serial measures of plasma or saliva cortisol for the following hour. Of course, the long intervening time from stimulus to hormone response of cortisol suggests it may represent more of a ‘summation’ or integration of multiple psychological events during this time, which thus complicates efforts to relate subsequently activated cortisol levels to specific brain activity at the time of the stressor. The more rapid responses in the case of ACTH that can be measured in plasma provides a shorter interval between brain events and hormone response, and may allow designs in which several neurosecretory responses can be tested within a single paradigm.

Lifestyle, Everyday Stress, and Gender considerations—LHPA axis basal function as well as stress responsiveness is known to be affected by a large number of biological,

sociodemographic and lifestyle factors including age, gender, sex steroid levels, pregnancy, lactation, smoking, coffee, and alcohol consumption (Kirschbaum et al., 1999; Kudielka et al., 2009; Steptoe et al., 1998). Psychological variables such as “everyday stress” and “hassles” also appear to have significant influences on circadian and stress-responsive cortisol (Kudielka et al., 2009; Steptoe et al., 2000; Steptoe et al., 1998), with significant variability between workdays vs. weekends (Kunz-Ebrecht et al., 2004; Liberzon et al., 2008). LHPA axis function is also thought to have genetic contributions (Wust et al., 2000) and is also affected by life history such as early life trauma (Heim et al., 2001), and chronic stress (Ockenfels et al., 1995). Each of these factors could lead to substantial inter- and intra-individual differences, and as they may be variables of interest, or nuisance factors which could seriously confound a study design, all of these need to be taken in to consideration and/or controlled as much as feasible for when designing studies to link LHPA axis function to brain activity.

Cigarette smoking in particular also has strong effects on basal and stress responsive LHPA axis function (Kirschbaum et al., 1992; Steptoe and Ussher, 2006). Nicotine can directly stimulation of CRH via cholinergic receptors, and acutely, cigarette smoking can lead to increased salivary cortisol (Kirschbaum et al., 1994; Kirschbaum et al., 1992). Habitual smoking is associated with higher levels of circadian cortisol (King, 2008; Kirschbaum et al., 1992; Steptoe and Ussher, 2006), blunted cortisol responses to psychosocial stressors (Kirschbaum et al., 1993b), and elevated cortisol responses to awakening (CRA) (Steptoe and Ussher, 2006), acute nicotine abstinence has variable effects on these alterations. Therefore, controlling for habitual cigarette smoking is an important consideration in any study of LHPA axis function; and acute abstinence from smoking by habitual smokers will not be a sufficient control. Eating or fasting also impacts plasma and salivary cortisol levels (Kirschbaum et al., 1997) with a reliable postprandial increase in cortisol (Brandenberger et al., 1982), and stress responsivity (Gonzalez-Bono et al., 2002) and thus time from last meal should be considered and controlled for both in measurements of ‘basal’ circadian cortisol, as well as in design of stress-response paradigms. Caffeine has well-documented effects on the BOLD signal (Laurienti et al., 2002; Liu et al., 2004), and may also stimulate LHPA axis output acutely, although the data are somewhat mixed (al’Absi et al., 1998; Kunzel et al., 2003; Lin et al., 1997; Lovallo et al., 2006). Alcohol, both acutely (Dai et al., 2002) and chronically (Gianoulakis et al., 2003) alters circadian LHPA axis and responsiveness. Thus it would appear that the common practice of asking imaging subjects to abstain from alcohol and caffeine for 24 hrs before the scan may also help control for potential effects on LHPA axis; and it will also be important to consider habitual smoking and drinking patterns in recruitment plans or as potential covariates. Interestingly, presence of nausea can have effects on cortisol as well (Otto et al., 2006). Thus the large number of potential influences complicate efforts to control “incidental” influences on LHPA axis function, but should be carefully controlled for as feasible.

If studying female subjects or both sexes, it is very important to assess and control for the time in the ovulatory cycle in females. Estrogen also has known effects on HPA axis function, including on gene expression of CBG (Wiegratz et al., 2003), and may have effects on other systems as well. Cortisol stress responses in healthy women in the luteal phase are comparable to those in men, but women have significantly lower cortisol responses when in the follicular phase or are taking oral contraceptives (Kirschbaum et al., 1999; Rohleder et al., 2001; Wolf et al., 2001), which appear to affect cortisol binding globulins (CBG) (Wiegratz et al., 2003). Therefore, studies including men and women should assess women while in the luteal phase and not taking oral contraceptives. Pregnancy has profound effects on LHPA axis function, including increased circulating cortisol (Scott et al., 1990) and very high levels of CRH (from placenta (Campbell et al., 1987)) especially after the second trimester, and thus it is important to control for current reproductive status.

Circadian considerations—The LHPA axis, as well as several other neuroendocrine systems (e.g. growth hormone (GH) and melatonin systems) have circadian rhythms, and thus time within the circadian cycle must be controlled for. ‘Basal’ plasma ACTH and cortisol levels peak in the morning and have a nadir in the evening (Czeisler and Klerman, 1999; Linkowski et al., 1993), thus it is important that the time of the day when the hormone sample is collected (or hormone dose is administered) remain constant. For study of psychological activation of neurosecretory systems, afternoon is best for study of activations, as the system is more sensitive during the nadir (less negative feedback due to circulating hormone level). It might be also useful to assess normal circadian rhythms (magnitude and slope of the diurnal variability) of the subjects, in the case of cortisol. While circadian cortisol appears to have trait-like stability, some (Hellhammer et al., 2007; Stone et al., 2001) have suggested that measurements over multiple days are necessary to reliably assess measures of diurnal cortisol and response to awakening, because cortisol can also be subject to a number of daily psychological and physiological influences. This can be accomplished in the subjects homes by taking 3–5 time point saliva samples for several days before the scan, to both determine the person’s “normal” patterns of diurnal variability, and ‘expected basal’ values at time of the scan. Obviously this will add cost and effort to these studies, and it should be noted that so far no reports demonstrated differences between the measures from the day immediately prior to scan and the multi-day averages; such data will be useful to plan future studies.

Time and frequency of sampling in paradigm

Measures of “Basal” and circadian hormone levels—Various measures of circadian rhythms can be used as regressors of brain activity in between-subject analyses. “Baseline” level (e.g. at a morning time point, or immediately before the start of the experiment) can be used as regressors of interest, and have been linked to hippocampus and ACC activity (Bonne et al., 2003). The diurnal variability, e.g. morning (peak) salivary cortisol level minus evening (nadir) salivary cortisol level, or approximations of the slope of the linear portion of diurnal variability curve (ca. morning peak to evening nadir) can also be used; again samples can be obtained over several days at home to control for day-to-day variability. In order to minimize the influence of recurring events at particular times (e.g. lunch, etc.), saliva samples can also be collected at random times over several days (Hellhammer et al., 2007; Stone et al., 2001; Urry et al., 2006).

Human cortisol levels continue to rise as much as 50% in the first 30–45 min after awakening (Kunz-Ebrecht et al., 2004; Wust et al., 2000). This “cortisol response to awakening” (CRA) is a relatively recently described phenomena, which may provide a measure of the sensitivity of the HPA axis to a naturalistic psychosocial stressor (awakening and getting up in the morning, for review see (Fries et al., 2008)). The CRA can be effectively characterized as the area under the curve (AUC) of as few as three saliva samples; i.e. asking subjects to collect saliva immediately upon waking (before getting out of bed), and again at 30 min and 45 min after awakening (Edwards et al., 2001; Fries et al., 2008). To avoid effects of plasma contamination and postprandial cortisol effects, it is important to ask subjects not to brush their teeth or eat during this period (Fries et al., 2008), and again obtaining measures over several days is important to control for day to day variability.

Measures of Hormone Responses—Plasma or saliva hormone concentrations during an activation protocol can be used as regressors of brain activity in within-subject analyses. Because hormone concentrations often are skewed to the right and do not display a normal distribution, it is often appropriate to perform log (or natural log) transformation. Hormone response measures that could be used as regressors for within- or between-subject analysis include the maximal response (i.e. maximal concentration following stimuli minus pre-stimulus ‘baseline’), response at a given time point, and an integrated measure of the summated

response over time, the area under the curve (AUC). AUC provides an integrated measure of hormone response that is a widely used to analyze both circadian and acute response data. Pruessner et al. (2003) has provided a useful summary and derivation for two AUC measures: AUC with respect to ground (AUC_G), which includes the total differences between subjects (including baseline differences, formula:

$$AUC_G = \left(\sum_{(t=1)}^{(n-1)} (m_{(t+1)} + m_i) / 2 \right)$$

and AUC with respect to increase (AUC_I which is baseline subtracted and thus represents only the response over baseline, formula:

$$AUC_I = \left(\sum_{(t=1)}^{(n-1)} (m_{(t+1)} + m_i) / 2 \right) - (n - 1) * m_1$$

The time-course of the hormone response to the given stimuli/behavioral activation paradigm will determine the schedule of specimen collection, as well as the number of individual activations that can be measured within a given neuroimaging paradigm.

Psychological/behavioral ‘activation’ paradigms for inducing endogenous neuroendocrine responses

Accumulating evidence suggest that HPA axis activation is under the influence of fairly specific social and contextual stimuli, rather than being driven by general ‘distress’ or by negative affect. A large meta-analysis of over 200 studies of cortisol and ACTH responses to acute psychological stressors, including passive and motivated ‘performance’ tasks, found wide variability in response across paradigms (Dickerson and Kemeny, 2004). Only tasks which included social-evaluative “threat” (i.e. task performance could be negatively judged by others) and/or “uncontrollability” were reliably activators of the HPA axis responses when methodological factors and other stressor characteristics were controlled for (Dickerson and Kemeny, 2004). In marked contrast, ‘passive’ tasks, including viewing highly aversive pictures, and various “mood induction” paradigms, and even paradigms associated with considerable distress by self report, such as exposure to phobic stimuli (Curtis et al., 1978; Knopf and Possel, 2009) do not reliably activate LHPA axis responses (Dickerson and Kemeny, 2004). Tasks containing both social evaluative threat and uncontrollability achieve the highest levels of activation. The most widely used (and probably most reliable) paradigm of this type is a several part motivated performance task known as the Trier Social Stress Task (TSST) (Kirschbaum et al., 1993a), which involves 15 minutes of psychosocial stress, including delivering a personally-salient public speaking task (e.g. mock job talk) to an audience of non-responsive judges (and often a video camera), followed by a mental arithmetic task (serial subtractions by 13 from a four digit number) with critical feedback. The TSST induces significant and reliable responses in plasma ACTH, plasma and salivary cortisol, and cardiovascular responses (Kirschbaum et al., 1993a; Kudielka et al., 2009; Kudielka et al., 1998), making it a useful and widely replicated LHPA axis activation paradigm. With respect to neuroimaging, however, the speech components might be less compatible with fMRI as they could introduce unacceptable head movements, although a public speaking task has been reported in a PET study (Tillfors et al., 2002) and a mental arithmetic task including verbal responses in an arterial spin-labeling study (Wang et al., 2005).

Efforts have been made to devise LHPA axis activation paradigms for the scanner environment that do not involve verbal responses. The “Montreal Imaging Stress Test” (MIST) (Dedovic et al., 2005; Pruessner et al., 2008) is one such adaptation of TSST mental arithmetic that uses a graphic user interface and manual responses to arithmetic problems. Difficulty level is individualized and presentation is time limited (and set to 10% shorter than the individual’s average time of completion). Social evaluative information is presented in the form of graphical display of individual vs. “normal” performance on the math tasks. This paradigm has induced significant cortisol responses in about half of subjects in original report (Dedovic et al., 2005; Pruessner et al., 2008).

Finally, populations with “sensitized” neuroendocrine responses may provide model systems in which to more readily study neural correlates of psychological effects on peripheral humoral factors. Sensitized LHPA axis measures have been seen in trauma-exposed individuals, including combat veterans and adult survivors of childhood abuse, including increased dexamethasone feedback sensitivity (Yehuda et al., 1993), and increased cortisol (Elzinga et al., 2003) and ACTH responses (Heim et al., 2001). We have taken advantage of sensitized responses to personal trauma recall to study neural correlates of HPA axis activation (ACTH responses to trauma recall) (Liberzon et al., 2007) also see King et al. in this volume). Another example of “sensitized” populations which have been used for studies of neuroendocrine studies include asthma patients, who mount a reliable immune response to presentation of allergen which may be modulated by psychological inputs (Rosenkranz et al., 2005).

Methods for drug/hormone administration

For studies of potential effects of hormones on specific sites of brain activity, the method of administration, the dosage, and the timing of the administration of hormone/agonist, or antagonist, will depend the pharmacokinetics and pharmacodynamics of the specific agent used. In the case of cortisol (and other steroid hormones) which readily cross the blood-brain barrier, oral or transdermal route can be conveniently used for chronic (over days) or acute (1 or 2 hours before scan) administration. For measurement of the potential ‘rapid’ non-genomic effects of cortisol or neurosteroids, or of secretagogues, administration via an indwelling catheter might be more appropriate. Some small peptide molecules can be administered intranasally, for example, intranasal oxytocin has been administered in neuroimaging studies by an intranasal bulb, with positive pressure administered to the nasal passage within minutes of the scanning sessions (Kirsch et al., 2005). When designing exogenous administration paradigm, the status of the endogenous hormones (e.g. circadian rhythm, etc.) is important to consider. It is also important to understand the relative number and affinity of endogenous receptors.

Discussion

Basal LHPA function appears to be relatively stable, trait-like (Huizenga et al., 1998), likely heritable, and under moderate genetic influence (Linkowski et al., 1993; Wust et al., 2000), and is also substantially modulated by ‘psychological’ factors, including chronic stress, early life adversity, psychopathology, etc (Johnson et al., 2008; Kuehner et al., 2007; Kunz-Ebrecht et al., 2004; Ockenfels et al., 1995; Steptoe et al., 2000; Wust et al., 2000). The mechanisms underlying these phenomena have not yet been delineated, but would appear to involve neural inputs impinging on and modulating systems controlling the timing and the magnitude of the diurnal variability (i.e. SCN). The convenience of saliva cortisol assays makes it feasible to measure “basal” and circadian cortisol function over several days at home before a scanning session that may prove useful at elucidating the effects of “chronically” dysregulated circadian rhythm in psychopathological states.

One measure of basal cortisol function is the slope of diurnal cortisol curves (which can be constructed from circadian salivary cortisol measurements at home). Two recent studies have examined diurnal cortisol slope and brain activity during emotional tasks in fMRI studies. Urry et al., 2006 found the slope of diurnal decrease in saliva cortisol to be positively correlated to mPFC and negatively correlated to amygdala activity during an effortful emotion regulation task in between-subjects correlation (Urry et al., 2006) suggesting a relationship between cortico-limbic regulation and circadian cortisol rhythm. Similarly, (Cunningham-Bussell et al., 2009) found a negative correlation between diurnal cortisol variability and amygdala and hippocampal activations to distressing pictures. Cortisol response to awakening, recently reported to be related to stress and psychological features (Fries et al., 2008) might also be also used to identify brain activity correlated with baseline activity of LHPA axis. A number of recent studies have correlated CRA to neuroticism, depression (Huber et al., 2006; Kuehner et al., 2007), PTSD (Johnson et al., 2008), and the stress of seafaring on a sailing vessel (Liberzon et al., 2008). A study by (Pruessner et al., 2007) reported that hippocampal size was positively correlated with CRA in humans, but to our knowledge there have not yet been function studies linking CRA to specific brain activity in humans. Measures of baseline LHPA axis such as diurnal slope and CRA can be related to brain function and these relationships examined among groups with differing levels of psychosocial stress or psychopathology.

LHPA axis reactivity to psychosocial stressors also appears to be under the influence of both genetic (Federenko et al., 2004; Wust et al., 2004) and developmental (Heim et al., 2001) factors. Epigenetic mechanisms for the developmental modulation of LHPA reactivity has been described in rodent models (Meaney et al., 2007). Studies to correlate peripheral LHPA axis 'output' (i.e. plasma ACTH and plasma or saliva cortisol) with specific brain activities during the activation paradigm will be helpful for identifying the specific neural circuitry that processes and identifies the relevant social and contextual cues associated with LHPA axis activation (i.e. social evaluative threat, non-controllability, and novelty), as well as those inputs that can lead to cognitive modulation, dampening, or "containing" LHPA axis responses.

Between-subjects correlations of the magnitude of stress-induced cortisol (AUC) and/or ACTH responses to brain activity at the time of the activating stressor can be used to investigate neurocircuitry potentially involved in the initiation of the neurosecretory response, but this approach contains several caveats. There are likely to be multiple intervening processes after the brain activity during the "activating stressor" and the rise in salivary cortisol ~35 minutes later, that may substantially influence the subsequent hormone response, but that are not captured by the information in the 'stress' scan. While use of plasma ACTH, which peaks at 5–10 min in plasma, allows for closer temporal relationships to brain activity which may provide better resolution and may be less confounded, a large number of neural events that may affect neurosecretion patterns can still occur in 5 min. Between subjects correlational approaches assume a 'linear' relationship between brain activations and subsequent ACTH or cortisol output in plasma; such a relationship may or may not exist, as suggested by two recent studies (Pruessner et al., 2008) King et al., this issue). Of course, the general problem of correlational studies that it is not possible to directly infer causation from correlation. Thus any observed positive correlations with cortisol AUC for example could represent both brain regions involved in activation of LHPA responses, as well as brain regions which actually inhibit responses, but come online at the same time as activations to help 'contain' the response. However, a correlation could also identify false positives - brain regions that are activated in a temporally coordinated fashion, but have nothing to do with LHPA axis control. If there is sufficient variability in LHPA axis responses to a particular activation paradigm, an alternative analyses is contrasting "responders" vs. "non-responders" to the paradigm. Such analyses are not limited to assumption of a linear relationship between activity and magnitude of response, and may be suggestive about the directionality of effects (i.e. higher activity of a brain region in non-responders is suggestive of inhibitory action), however, the general caveat of potential

false positives and inability to make strong inferences about co-occurring phenomena still applies.

A variation on this approach is to take multiple scans in tight temporal correlation with peripheral measures of cortisol or ACTH before, during, and after (recovery) the effects of a psychological activation paradigm. Performing contrasts at different time-points in the response can presumably measure the changes in brain activity that occur over time during each of the ‘phases’ of the hormonal response – i.e. from the immediate pre-response “latency” (1–2 min), to the beginning of the increase in ACTH in the bloodstream (2–5 min), to the ACTH peak (5–10 min), to the beginning of the increase in cortisol (10–15 min), etc. Brain activations at each of these time points (contrasted with pre-stress or other time-point scans) can then be correlated with subsequent hormone concentrations. Presumably brain activations at the early phases have the most to do with the LHPA axis response, whereas brain activations at subsequent time points may relate to termination of the response, and might also reflect the action of cortisol ‘feedback’ on the brain. It is not completely understood, for example, whether termination of LHPA axis responses is a ‘passive’ event following the termination of excitatory stimuli, or whether active inhibitory inputs terminate the neurosecretory response. Clearly there is inhibitory glucocorticoid feedback at the level of the pituitary and hypothalamus, and probably other brain regions such as hippocampus, but the potential neural basis of “fast” glucocorticoid feedback, as well as non-glucocorticoid mediated inhibitory inputs that contain responses is yet to be examined in humans. Evidence from rodent models suggests inhibitory influences on psychological LHPA axis responses from both hippocampus (Jacobson and Sapolsky, 1991) and medial frontal cortex (Diorio et al., 1993; Furay et al., 2008; Sullivan and Gratton, 2002). (Diorio et al., 1993) found that rats with lesions in mPFC/cingulate gyrus had similar peak levels of ACTH and cortisol immediately following a “psychological” stressor (20 min restraint), but hormone levels were slower to return to baseline, effects which were not seen in ether-induced hormone responses. Furthermore, cortisol implants into cingulate gyrus decreased ACTH and cortisol responses to restraint but not ether. Thus these data thus provide evidence that mPFC/cingulate gyrus is a site of glucocorticoid feedback on LHPA axis activations to psychological (but not neurogenic) stimuli in rodents; data from two recent studies in humans also implicate the mPFC/cingulate gyrus (Pruessner et al., 2008), King et al., this issue), consistent with these rodent findings.

The approach of studying brain activation to single activation episode however significantly limits the amount of the neuroimaging data that can be acquired from a single subject. A repeated measures design also allows for analyses of covariance (ANCOVA) using hormone level as a repressor of interest, which may reveal brain regions whose activity is predicted by endogenous circulating cortisol. Administration of exogenous cortisol in the context of neuroimaging is one way to elucidate the neural targets that mediate the psychological effects of glucocorticoid actions in humans. The presence of multiple receptor types, tissue-specific glucocorticoid response elements and receptor promoters, and specific distributions in the brain suggest complex and brain region-specific effects. There is evidence from animal models of differential effects of glucocorticoids providing inhibitory effects on hippocampus and stimulatory effects on amygdala, for example.

Specific GR- (e.g. mifepristone) and MR- (e.g. spironolactone) antagonists that can be safely administered to humans do exist (Clark, 2008; Young et al., 2003), although, we are not aware of any reports to date that have utilized these agents in neuroimaging. Such agents may help to delineate the specific contributions of the GR and MR receptor subtypes on brain activity. For example, basal and stress-activation patterns of brain activity can be examined after administration of GR- or MR-specific antagonists in neuroimaging paradigms, and specific activation of only one receptor type can be examined administration of cortisol following blockade by a receptor specific antagonist. Other existing pharmacological agents are available

to inhibit adrenal steroidogenesis metyrapone) and thus ‘clear’ the system of endogenous cortisol, as well as synthetic glucocorticoid agonists (e.g. prednisone, dexamethasone, etc.) with specific pharmacodynamics are available which may facilitate addressing specific questions. Imaging paradigms that study both the acute (hours) and chronic (days or weeks) administration of cortisol can be performed with cortisol and placebo, followed by neuroimaging paradigm that probes appropriate activity through emotional, memory, or stress, etc. tasks. Since synthetic glucocorticoids are known to have effects on cardiac function and hemodynamics (Ostergaard et al., 1999), it is important to ascertain that any observed effects are not due to non-specific effects on global cerebral perfusion; however, data from our group found no effect on HRF of chronic (25 mg cortisol for 10 days) or acute (a single 25 mg or 100 mg dose 1 hour before the scan) administration of cortisol. Functional neuroimaging affords the opportunity to directly detect potential rapid “non-genomic” effects on neurons that occur within minutes or seconds, and even further elucidate the nature of the membranous receptors, which have been proposed to be MR or MR-type receptors (de Kloet et al., 2008). Such studies will require rapid infusion of glucocorticoids by intravenous access, potentially in conjunction with administration of specific GR and MR antagonists (Young et al., 2003).

In summary, as of yet relatively few neuroimaging studies in humans began to examine brain function and basal and circadian LHPA activity (Bonne et al., 2003; Cunningham-Bussell et al., 2009; Drevets et al., 2002; Urry et al., 2006), the neural circuitry underlying activation of the LHPA axis (Liberzon et al., 2007; Ottowitz et al., 2004; Pruessner et al., 2008; Wang et al., 2005) and King et al., this issue), and the effects of cortisol administration on brain activations (de Quervain et al., 2003). Assessing the LHPA axis or stress axis function in functional neuroimaging environment is not trivial however, and requires in-depth understanding of psychology, LHPA physiology, neurobiology and neuroimaging because the system is complex, has baseline and reactivity characteristics, an internal rhythm and multiple self-regulatory feedback loops. On the other hand, growing sophistication in the analytical approaches to neuroimaging and neuroendocrine data, the recent availability and relative convenience of neuroendocrine assays, and growing number of investigators entering the field carry a great promise for rapid progress in addressing critical questions of neural control of stress response and of stress modulation of brain function.

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