TORC2 Reactivity to Acute Psychological Stress and Tonic Glucocorticoid Secretion

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TORC2 Reactivity to Acute Psychological Stress
and Tonic Glucocorticoid Secretion

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Abstract

Psychological stress induces activation of a highly developed but conserved physiological system called the hypothalamic-pituitary-adrenal (HPA) axis, which is ultimately regulated by glucocorticoid negative feedback. The HPA axis is active in both a tonic way, manifested as a diurnal hormonal secretion pattern, and in an acute way, as a phasic, stress-responsive surge in hormones. In addition to eliciting hormonal responses, stress elicits within the HPA system various genomic alterations, like regulation of expression of the corticotropin-releasing hormone (crh) gene in the paraventricular nucleus of the hypothalamus. Moreover, glucocorticoid negative feedback also entails regulation of crh gene expression. However, the specific mechanisms by which stress and glucocorticoids regulate crh gene expression are largely unknown. A proposed coactivator for the crh gene transcription factor CREB is a class of proteins known as transducer of regulated CREB activity (TORC) proteins, especially the hypothalamic isoform TORC2, which have been shown in vitro to be highly responsive to cellular stimulation. However, studies within live animals regarding TORC proteins are limited, and the goal of this project was to investigate TORC2’s responsiveness to both acute psychological stress and tonic secretion of glucocorticoids in rats. Although preliminary evidence was convincing, there was no effect for either acute stress (15 minutes restraint-stress) or tonic glucocorticoid secretion on TORC2 activity, while hormonal data indicated typical HPA axis responses to these manipulations.
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Introduction

Many of us recognize stress as an inconvenient emotion we have to endure, but it is actually a product of a highly developed and regulated physiological system that has critical purposes for survival. This system utilizes the hypothalamic-pituitary-adrenal (HPA) axis to exert its effects. In the natural world, animals are faced with stressors such as escaping from a predator or surviving starvation. Humans, on the other hand, experience a variety of complex stressor types, many of which are psychologically based. Chronic psychological stress is the form we are familiar with, and the type most people experience on a daily basis. We are faced with problems such as how to provide for our families, performing intellectually challenging tasks for work or school with limited time, or the various social pressures we knowingly or inadvertently worry about. Acute psychological stress is distinct from chronic stress in that it is a relatively short-term occurrence with ranging intensities – from mild stressors, such as performing mathematical tasks with a time constraint, to extreme stressors such as war combat, rape, or the death of a family member.

Both chronic and acute stress can interfere with healthy HPA function and can have deleterious effects on the body. Acute stress in particular can contribute to various psychological conditions such as post-traumatic stress disorder, anxiety, and major depressive disorder (1,7). Often these disorders are characterized by imbalances in HPA axis-controlled hormones, particularly a class of steroid hormones known as glucocorticoids, (cortisol in humans and corticosterone in rats, abbreviated CORT). Glucocorticoids are the primary effector hormones of the HPA axis, and while their secretion is highly regulated, they exert regulatory influence over the HPA system – hence this regulatory pattern has a cyclical mechanism. Although some of the forms of regulation (such as negative inhibition of releasing hormone) are understood, the mechanisms and anatomical sites of regulation have not been well defined. In order to address the dysregulation occurring in individuals with HPA axis disorders, it is preeminent that these mechanisms are understood. This project addresses some of these HPA regulatory mechanisms at the level of the hypothalamus.

The purpose of this project was to observe how various components of the HPA axis respond to and are regulated by both tonic and phasic secretions of glucocorticoids. A particular protein, TORC2, is a coregulator for gene transcription in the hypothalamus and is the central focus of this project. Research on TORC2 in vivo has been limited, however some components of
its behavior have been established in vitro. We expected to see that it would be responsive (activated) by 15 minutes of acute psychological stress in laboratory rats, but that it would not be as sensitive to removal of tonic secretion of glucocorticoids by adrenalectomy. However, the data collected from this experiment did not show any significant differences between test groups.

**Background**

*Stress response anatomy, physiology and the HPA axis*

There is a marked distinction between physical stress, in which an injury or illness alters a homeostatically regulated setpoint (such as blood volume), and psychological stress, in which higher brain centers of an organism interpret a situation as a threat to its well-being (23). Psychological stress is utilized in this project because it is more relevant to human circumstances and disorders, as the majority of the stressors that humans experience is not physical, but psychological. Physical stress, therefore, is not within the scope of this paper and all further discussion will be in the context of acute psychological stress.

Almost immediately upon the organism's perception of a stressful event, a multitude of physiological changes take place in order to assist the animal in coping with the challenge. The first wave of response occurs through activation of the sympathetic branch of the autonomic nervous system, and is both rapid and transient. Postganglionic sympathetic innervation causes an increase in heart rate, induces the pancreas to release glucagon into the bloodstream, and stimulates the adrenal medulla, increasing secretion of catecholamines (norepinephrine and epinephrine), which also generate similar outcomes as sympathetic innervation (23). In addition, catecholamines affect peripheral and central vasculature, causing vasoconstriction of digestive and neural tissue vessels, while enlarging the vessels of skeletal muscles. Increased blood flow to skeletal muscles coupled with increased heart rate and blood glucose levels enhance the chance of an animal's survival in dangerous situations by diverting energy to appropriate tissues. The sympathetic nervous system is vital in the stress response because it drives these variables away from homeostasis in order for the animal to escape harm. The HPA axis is simultaneously activated during stress, and although it has some influence during the immediate stress, its primary responsibilities are to return the organism to homeostasis and prepare it for future stress (23).
HPA axis activity begins when complex interactions of neural pathways activate neurons in the hypothalamus. The hypothalamus acts as an integrator for information received from all over the brain, as well as peripheral sensory information regarding conditions of both the internal and external environment. It is heavily involved in osmoregulation, growth, metabolism, reproduction, circadian rhythms, and is a major component in the execution of stress responses (25). Initiation of the psychological stress response is elicited when the organism interprets a situation as threatening, a process occurring in higher (more evolutionarily developed) brain centers (8). The initial perception of stress is probably accomplished by the prefrontal cortex (i.e. optical or olfactory stimuli), and is then transmitted as a neural signal through a network of other brain regions such as the hippocampus and the amygdala, though these pathways are largely unknown (21) (Figure 1). However signal transduction occurs, once the situation is interpreted as being stressful, the HPA axis is activated, beginning with excitation of a specialized class of neurons in a sub-region of the hypothalamus called the paraventricular nucleus (PVN) of the hypothalamus, which is located bilaterally adjacent to the third ventricle (Figure 2).

These specialized cells, located in the medial parvocellular region of the PVN, produce corticotropin releasing hormone (CRH), a peptide hormone instrumental in transduction of a stressful stimulus into a physiological response. In concurrence with neuronal depolarization, 2nd-messenger pathways are also activated, some of which actually results in increased transcription of the crh gene (15,16). Proteins mediating this genomic process are cAMP-response element binding (CREB) protein and transducer of regulated CREB activity (TORC) (this process will be discussed in detail in later sections of the paper). CRH neurons have axonal projections to the median
eminence, the superior portion of the neurohypophysis (20). Here, previously synthesized CRH peptide is stored in vesicles, ready for release into the hypophyseal portal system. Upon cellular excitation, specifically depolarization, CRH is released into the portal blood and travels to the pars distalis region of the adenohypophysis (also known as the anterior pituitary in most mammals), and stimulates corticotrophs to release ACTH into systemic circulation (20). The primary function of ACTH is to stimulate the production and release of steroid hormones called glucocorticoids, namely CORT, from the cortex of the adrenal glands. Glucocorticoids can cause changes that enable the organism to react to the immediate stressor, but are heavily involved in shutting off the stress response and returning the organism to homeostasis through negative feedback inhibition (23).

**Glucocorticoids: mechanistic properties, classes of action, and tonic versus phasic secretion**

As mentioned, the primary role of glucocorticoids is to terminate the stress response and to restore homeostasis. They do, however, have some important roles in altering various physiological functions during stress, one of which is enhancing the secretion and activity of catecholamines (23). Glucocorticoids also have a role in sharpening cognition via increased cerebral glucose utilization and suppression of immunity, but they predominantly influence metabolic function (23). Glucocorticoids induce gluconeogenesis in the liver and increase catabolic breakdown of fats, protein and carbohydrates (20). However, most glucocorticoid effects are genomic in nature, therefore requiring activation of DNA transcription and de novo protein synthesis. The effects of glucocorticoids occur in a slower wave of events that follows the initial, sympathetic response to stress, somewhere within a time frame of approximately 1 hour after the onset of stress, enough time to allow for gene transcription and protein synthesis from mRNA (20,22). It is possible, even likely, that the animal has already evaded the stressor by this time. Therefore, many of the actions of glucocorticoids do not influence the immediate stress response, but instead return the animal to a baseline physiological state, and also enhance the chances of survival in a subsequent challenge.

Within the context of stress, glucocorticoid actions are separated into four distinct classes: stimulatory, suppressive, preparative, and permissive (18,23). Stimulatory and suppressive glucocorticoid actions are virtually instantaneous upon systemic release from the adrenal cortex; these actions can only be considered stimulatory or suppressive if they are
involved in coping with the immediate stress (23). These functions are ultimately related to acute secretions of ACTH, which directly induce secretion of glucocorticoids. Glucocorticoids inherently feed back upon the hypothalamus and pituitary gland to terminate the increased secretion of ACTH. However, levels of both ACTH and glucocorticoids return to basal levels by about 30 minutes after the termination of stress, and glucocorticoid negative feedback contributes to this timely shut-off of the response (5).

The latter two classes of glucocorticoid action exert their effects in a much slower manner. Glucocorticoid actions are considered preparatory if they enhance the animal’s response to a future stressor and do not aid the reaction to the present stressor. An example of this is that glucocorticoids induce the liver to increase its rates of glycogen synthesis, thus upregulating energy stores within the animal to aid the response to a subsequent stressor (23). Permissive actions of glucocorticoids are those that do not directly cause physiological changes, but enhance the effects of other systems or hormones. Permissive glucocorticoid actions are manifested through a circadian, diurnal cycle of secretion (23). A tonic surge of glucocorticoids occurs each day, peaking just prior to the start of the active period; for rats this occurs prior to lights off (Figure 3). It is thought that this tonic secretion ensures that the organism can manage metabolic variations that will occur once feeding begins (5). A relevant example of permissive action is the tonic regulation of ACTH secretion. In rats without adrenal glands, ACTH is significantly upregulated. However, replacement of circadian-cycle levels of glucocorticoids mostly normalized ACTH levels that are usually regulated by phasic increases in glucocorticoids (10).

**Regulation and negative feedback produced by the HPA axis**

Negative feedback has been mentioned in the context of HPA axis activity, and it is clear that there are several purposes for this feedback. Because the sympathetic nervous system (and certain components of the HPA system) drives homeostatically regulated variables away from...
their set points, there must be a mode of regulation in order to prevent overshooting the response, while still allowing beneficial activity to take place (23). In combination with reduced neural excitation of the PVN, a transient secretion of glucocorticoids, known as phasic secretion, is also vital in terminating the response (25). ACTH levels peak at around 15 minutes of acute psychological stress in rats, and are down to almost normal levels by an hour after the onset of stress (Figure 4). Again, the pattern of glucocorticoid secretion closely resembles that of ACTH secretion (but with a slight time delay), as their release is directly responsive to plasma ACTH concentrations. The phasic surge of glucocorticoids, coupled with a decrease in neural excitation, is responsible for the decrease in ACTH secretion and the subsequent depression of glucocorticoid levels, due to the negative feedback role of glucocorticoids on both the pituitary and the hypothalamus (3). It is also well established that glucocorticoids inhibit the both secretion of ACTH and transcription of the pomc gene, encoding the prohormone of ACTH (20). More importantly, glucocorticoids also inhibit crh gene transcription (5, 21). However, the mechanisms and location of feedback, both immediate and genomic, have been largely unknown; only recent research has delved into these mechanisms (Figure 5).

In addition to the phasic surge of glucocorticoids observed during acute psychological stress, tonic (diurnal, circadian) secretion of glucocorticoids has also been shown to influence various components of the HPA axis. Glucocorticoids exert their effects through binding to glucocorticoid receptors in the cytoplasm of cells within the target tissue. The receptors dimerize, transport to the nucleus, and likely bind directly to DNA to either elicit, or in the case of feedback, inhibit gene
transcription; gene expression inhibition occurs at a negative glucocorticoid response element (nGRE) (4,11,21). It has been widely established that removal of endogenous glucocorticoids increases both basal and acute levels of ACTH and corticosterone (5,22). Interestingly, when glucocorticoids were replaced in adrenalectomized rats (in the drinking water) in a manner that mimicked the circadian rise at the beginning of the active period but not phasic secretions, basal and stress-induced ACTH levels were largely normalized, indicating a permissive manner of glucocorticoid feedback (Figure 4). Girotti et al also showed that crh gene transcription is negatively regulated by glucocorticoids in a tonic manner (removal of endogenous glucocorticoids by adrenalectomy), as shown by alterations in levels of crh heteronuclear, or primary, RNA transcript (hnRNA) in the nucleus of CRH neurons (5) (Figure 6). This might occur through a negative response element sensitive to glucocorticoid inhibition, in which the dimerized receptor-ligand complex binds directly to the DNA and prevents further transcription (15).

Although some knowledge exists about where feedback occurs, it is likely that glucocorticoids inhibit cells both within and outside of the HPA axis (21). Intrinsic feedback, meaning regulation that occurs within tissues of the HPA axis, is characterized as regulation of one or more components of the HPA system. This indicates that feedback occurs within either neurons of the PVN, corticotrophs in the pituitary gland, or endocrine cells in the adrenal cortex, and is directly affecting these tissues. Studies about glucocorticoid effects on the adrenal cortex are limited, but research has shown that a large extent of feedback occurs at the level of the pituitary, and to some extent upon the hypothalamus (5,22). In the context of crh gene expression, this would likely mean that glucocorticoid feedback is occurring directly on CRH neurons of the hypothalamus, possibly through a transcription inhibitor (11).

On the other hand, there has also been evidence that extrinsic feedback regulates HPA axis activity. Extrinsic regulation in this case is defined as regulation of neural input to the
hypothalamus, specifically the PVN, thereby reducing cellular activity in the PVN. Neuronal excitation is preeminent for HPA axis activation (and crh gene transcription); therefore if neuronal excitation is inhibited by glucocorticoids, then it can be concluded that HPA function is regulated extrinsically, or in an indirect manner. It is likely that feedback occurs both extrinsically and intrinsically, and by several different mechanisms (21).

There is evidence that removal of tonic glucocorticoid secretion via adrenalectomy (ADX) does not increase basal levels of neuronal excitation in the PVN (as indicated by the cell-activation marker, c-fos mRNA, Figure 2), but drastically increases stress-induced cellular activity, suggesting that regulation of basal levels of cellular activity are not regulated by tonic secretion of glucocorticoids (Figure 7), but that there is an interaction of ADX and stress. In a separate study, Girotti et al used a restraint-stress time course to show that there is a rise and fall in c-fos gene expression, peaking at about 30 minutes and falling to basal levels by 60 minutes (22). It is important to note that this decrease of hypothalamic neural excitation occurs even if the stressor persists, indicating that while neural input and interpretation of the stress remains, hypothalamic cell activity decreases. This implies that the mechanism of terminating these temporary increases in gene expression occurs in an intrinsic manner (5). Crh gene expression follows a similar time course, except the peak of expression occurs at around 15 minutes and falls to baseline levels by 30 minutes.

However, it is important to realize that the modes of regulation for c-fos and crh gene expression are probably not the result of feedback by phasic secretion of glucocorticoids, because inhibition of transcription for both genes occurs in both adrenal-intact and adrenalectomized animals (22). Interestingly, Kageyama and Suda as well as Liu et al have suggested that this indicates intracellular mechanisms of feedback, which would probably occur through resident protein transcription inhibitors specific for crh gene (11,16). Conversely, it might indicate that feedback occurs extrinsically and independent of glucocorticoids. Clearly, there are many explanations for negative feedback, and these

Figure 7. In the AM cohort (gray bars), there is no difference in cellular activity between the no-stress conditions of either the sham or ADX condition, indicating a lack of basal cell-activity regulation by tonic glucocorticoid secretion. However, there is an interaction of ADX and stress (5).
mechanisms of gene expression feedback require further investigation. The genomic component of both HPA axis activity and regulation is central to this project, and it is important to discuss the mechanisms of transcription with perspectives on phasic and tonic actions of glucocorticoids.

**Crh gene transcription and TORC: regulatory mechanisms**

It is known that acute psychological stress induces notable increases in *crh* gene expression within the PVN; hnRNA levels peak at around 15 minutes of restraint stress and fall to almost basal levels by 30 minutes (5,15,22). While many of the processes of transcription are understood, what is unknown are the exact means by which cellular activity leads to induction and regulation of *crh* gene transcription.

An array of neural signals, from depolarization of the cell to activation of second messenger pathways, is required to initiate *crh* gene transcription (6). The protein kinase A (PKA) pathway, which utilizes cyclic-adenosine mono-phosphate (cAMP) and PKA, has been shown to activate transcription through phosphorylation of the transcription factor cAMP response element-binding protein (CREB) by PKA (15,16). It has been demonstrated in vitro in hypothalamic 4B cell lines that activation of CREB via cAMP pathway is necessary, but not sufficient, for *crh* gene transcription, measured by promoter activity of the *crh* gene (15,16,17,24). *In vivo* studies have indicated that there is an increase in *crh* gene expression that directly corresponds to phosphorylation of CREB upon stress induction (2,14). Clearly, phosphorylated CREB plays an integral role in *crh* gene expression; however CREB is involved in transcription of an immense number of genes within various tissues (24). Therefore, it likely requires a coactivator to induce specific genomic activity, and a class of proteins called transducers of regulated CREB activity (TORCs) are valid candidates (15,16,17,24).

There are three isoforms of TORC, all of which interact with pCREB and are proposed coactivators for pCREB activity (15,16). TORC proteins have been found extensively in the liver and shown to be actively involved in expression of gluconeogenic genes in hepatic cells, in cooperation with cAMP and therefore CREB (13). Although all three isoforms are present in the brain, TORC2 is the predominant species in hypothalamic tissue (particularly in the PVN), and appears most responsive to stimulation by the PKA-activating drug forskolin in vitro (16). Somewhat counter-intuitively, TORC proteins are sequestered in the cytoplasm as a result of phosphorylation by specific kinases, such as salt-inducible kinase and AMP kinase; this
phosphorylation increases its affinity with the scaffolding protein 14-3-3, which retains TORC in the cytoplasm (16,24) (Figure 8). Activation of the cell, specifically the cAMP pathway, activates PKA, inhibiting salt-inducible kinase, preventing tonic phosphorylation of 14-3-3, and allowing TORC to shuttle to the nucleus (24). A phosphatase is also probably required, and calcineurin (activated by calcium ion influx into the cell) is a likely candidate (24). Once TORC has translocated from the cytoplasm to the nucleus, it is able to bind to pCREB and initiate gene transcription at the crh promoter (16). However, it is unknown how this protein behaves in vivo in response to stressful stimuli and in the presence of tonic and phasic increases in glucocorticoids. The aim of this project is to observe TORC2 behavior in vivo, manipulating both tonic and phasic secretions of glucocorticoids.

**Preliminary studies**

I assisted with several preliminary studies exploring the feasibility of measuring TORC2 immunoreactivity in CRH neurons. The main measure of TORC2 activity is a quantitative analysis of the percentage of cells with nuclear (as opposed to cytoplasmic) TORC2 (Figure 10). In the first experiment, done by our lab’s postdoctoral fellow Michael Weiser, the nature of TORC2 was studied in organotypic hypothalamic cultured tissue to reaffirm the previously published in vitro findings concerning TORC2’s behavior in response to artificial cellular stimulation (16). Following this experiment was an in vivo pilot study to optimize both tissue fixation and staining conditions for TORC2. Although the test groups were relatively small (n = 3-4), some data were collected. These numbers were not highly conclusive, however we were able to make some inferences based on statistical trends.
Another pilot study was conducted using the newly established fixation conditions, in order to test and perfect the actual IHC procedure; it was also an initial test to explore the effects of stress upon TORC2 activity *in vivo*. In this experiment (n=4) TORC2 activation was compared between no-stress and stress conditions in adrenal-intact animals. Although this study did not yield statistically significant data, the trends were strong enough to move forward with investigation. These studies became the basis for the main experiment of this project, which looked at both tonic glucocorticoid actions and stress effects (phasic actions) on TORC2 activation.

**Preliminary Experiment 1: cellular stimulation in vitro induces nuclear translocation of TORC2**

TORC proteins were discovered relatively recently, and there have been a limited number of studies regarding its activity in the brain, especially *in vivo*. Liu et. al (2008) found that levels of phosphorylated CREB (thus activated) and levels of nuclear TORC2 both increased as a function of dose of the cell-stimulating (via PKA activation) drug forskolin administered to hypothalamic culture cells. This indicates that CREB and TORC2, hence *crh* gene transcription, are responsive to cellular activation of cAMP/PKA by forskolin. Primary neuronal cell culture studies by Liu et. al (2009, 2010) as well as several organotypic studies done within our lab (Spencer Lab, unpublished data 2010), have shown that TORC2 is highly responsive to the cell-stimulating drug forskolin.

The organotypic cell culture study within the Spencer lab (for which I was able to perform cell quantification and data analysis), is a foundation for both the preliminary and main experiments of this project. The study had a 2x2 factorial design with the independent variables being administration of CORT (500 nM) pretreatment (2 hours prior to other manipulations) or
forskolin (10 uM) onto the tissue; the negative control was vehicle, which was culture media. The tissue was exposed to neither CORT pretreatment nor forskolin, to one of the two, or to both CORT pretreatment and forskolin for 30 minutes. Upon stimulation by forskolin, CRH neurons showed a significant increase (P<0.01) in TORC2 activation, as measured by nuclear translocation (Figure 9, Figure 10). This procedure was also performed in the presence of CORT, and showed similar trends as in the absence of CORT. This indicates that TORC2 is not responsive to 2 hr of CORT pretreatment but is responsive to cellular stimulation by forskolin. If in vivo studies reflect the findings of this preliminary in vitro study, then TORC2 should be responsive to acute psychological stress (i.e. cellular stimulation of the CRH neuron), but not to phasic glucocorticoid negative feedback, and possibly not to tonic glucocorticoid secretion either.

Preliminary Experiment 2: perfusion vs. post-fixation as the tissue preservation method for optimal hypothalamic TORC2 immunostaining pilot study

The main purpose of this study was to enhance tissue fixation conditions as well as to begin optimizing TORC2 characterization. TORC2 has been consistently difficult to characterize, which has remained true throughout all of the studies for this project. There have been many difficulties with the fluorescence immunohistochemistry staining procedure (see methods) in regards to tissue integrity, high levels of background, and quality of the signal.

Paraformaldehyde (PFA) fixation of brains (as opposed to flash-freezing in isopentane) produces better image quality for TORC2, therefore it is the preferred method of tissue fixation. This experiment used two methods to seek an optimal tissue preservation method – paraformaldehyde vascular perfusion and tissue post-fixation. Both methodologies involve a PFA incubation period, the duration of which is discussed in the following paragraph. The difference between the two is that in the former method, 4% PFA solution is injected into a live, anesthetized animal via the left ventricle and allowed to disseminate into the capillary beds of all tissues (including the brain), thus preserving the tissue. Post-fixation, on the other hand, requires that the brain be extracted immediately post-decapitation, blocked (using a razor blade) around the hypothalamus, and placed into PFA (12).

Prior to this experiment, the best duration of fixation in PFA solution was established to optimize both tissue integrity and staining quality. The tissue must be well enough preserved that it can endure numerous washes and extended incubation periods in PBS-solutions required
for immunohistochemistry. If the tissue has not been fixed in PFA for long enough, it could degrade throughout the procedure (either partially or entirely), which makes cell quantification difficult. However, if the tissue is in PFA for too long, the amount of background in the signal is dramatically higher. Various durations of fixation incubation ranging from 4-18 hours have been tested in studies not mentioned in this paper. It appears that 8-10 hours yields the most optimal condition for staining while preserving tissue integrity, and this duration was utilized for both the perfusion and post-fixation procedures. The brains then underwent the normal post-fixation and immunohistochemistry procedures (see methods section for a detailed protocol), using both diaminobenzidine (DAB) immunoprecipitation and immunofluorescence dual staining.

Although vascular perfusion produced better staining quality and tissue integrity, it was later decided that the procedure produces too much of a confounding variable (for the control group) to be used in stress research. The anesthetization procedure was probably very stressful for the animals – the intraperitoneal injection or invasion of the chest cavity (both are physical stressors) could have activated the HPA axis; even pentobarbital (the drug used for anesthetization) is a potent activator of the HPA axis (26). This variable may have accounted for the large amount of variability in TORC2 immunoreactivity patterns within the perfused-group tissue, the data from which was not valuable because of this range of values. However, the post-fixation method utilized in this experiment, in which the brains were blocked around the hypothalamus and immersed in PFA for 10 hours, appeared optimal for both integrity and staining quality without significant stress to the animals. Cells for both DAB- and fluorescence-stained tissue were quantified and analyzed, and although the test groups were small, some useful and interesting data were gathered.

This experiment had a “pseudo”-2x2 design, which assigned 4 animals per test group (N=16) with independent variables of perfusion and 30 minutes of restraint stress (it is important to note that we were not looking for significant differences or an interaction between groups – it was for the purposes of exploring fixation methods only). The reason stress was included as an independent variable was to ensure that the TORC2 staining for both home-cage and restraint-stress conditions were optimal. While the original groups contained 4 animals each, there was another unintentional variable that was not discovered until analysis: the time of day when the brains were harvested (the experiment took place between 1200 and 1600h). The animals sacrificed towards the end of the testing period were killed closer to times that approach
the circadian elevation in glucocorticoid secretion. This became apparent when the groups were split between brains harvested before and after 1400h. While this variable was accidental, there was a surprising difference in TORC2 activity between the two time points. Because time of day came to be viewed as an additional independent variable, the group sizes were reduced to only 2 animals, which unfortunately do not have a high level of statistical power. Even so, there were some interesting, statistically significant differences between test groups.

Before discussing these differences, it is important to appreciate the distinction between DAB and fluorescence IHC in this study. In the DAB-stained sections, the entire PVN, except magnocellular neurons, was quantified, while in fluorescence IHC, only CRH-positive neurons were analyzed. The data actually did diverge between these two methods; the fluorescent sections did not yield statistically significant differences between any groups, while the DAB-stained sections did. For the DAB sections, there was a difference observed between both time of day (both groups were of the home cage [HC] cohort) and restraint-stress (all before 1400h to eliminate this variable)(P<0.05 in both instances)(Figure 11). Although the statistical power for these groups is limited, they were statistically significant, which encouraged further study of stress-induced TORC2 nuclear translocation.

**Preliminary Experiment 3: Effects of 15 minutes acute restraint-stress on TORC2 activity in the PVN**

This experiment was designed to fine-tune the IHC staining procedure, and concurrently seek evidence that supports TORC2’s responsiveness to acute psychological stress. The experimental design was simple; there were only two groups with no stress or 15 minutes of restraint stress (see methods) as the independent variable (n=4). There were four separate test runs of the IHC procedure on non-PVN tissue sections (selected randomly) and two on PVN-containing sections from all animals in the study. Once the staining procedure was optimized,
there was a final run that included all animals and PVN containing tissue sections. Data (Figure 12) from this experiment do not show a significant difference between test groups, but there was a strong trend (p=0.067). Although the data were not significant, it seemed viable that this was due to the fact that the group sizes were not large enough to yield sufficient statistical power. Consequently, this trend was strong enough to suggest that it is likely in a larger population set that there would be statistically significant differences in percent nuclear TORC2 between stressed and non-stressed animals, the measure investigated in the main experiment of this project.

Pre-experiment summary and primary study hypothesis

The HPA axis is regulated on several time scales and through various mechanisms. Genomic activity, specifically crh gene transcription, is rapidly increased in response to stress and subsequently inhibited by glucocorticoids, but the details of these mechanisms are not understood. The phosphorylated form of CREB is required but not sufficient alone to elicit crh gene transcription, and TORC2 is a likely candidate as a coactivator of CREB. TORC2 has been shown to be responsive to artificial stimulation of the cAMP pathway in vitro, as measured by an increase in nuclear TORC2 under this condition, as well as by its necessary participation in crh gene promoter activity (16). This type of cellular stimulation also occurs upon perception of stressful stimuli by an organism; therefore TORC2 should be activated by acute psychological stress.

In a preliminary in vitro study described above, TORC2 appeared unresponsive to 2 hr CORT pretreatment, indicating that TORC2 may not be regulated by a short-term (phasic) increase in glucocorticoids. However, research has indicated that there are differences, particularly upregulation, in crh gene expression upon the removal of tonic glucocorticoid secretion (5). The removal of the adrenal glands caused an increase in both basal and stress-induced levels of crh gene transcription (Figure 6).

If TORC2 were contributing to crh gene transcription, then removing the adrenal glands would likely produce an increase in TORC2 activity in CRH neurons in both stressed and non-
stressed groups. In the main study, we anticipated seeing an increase in percentage of nuclear TORC2 in CRH neurons with acute psychological stress, an increase in nuclear TORC2 in basal levels of ADX animals, and a likely increase in stress-induced TORC2 activation of ADX animals compared with all other groups, all of which reflect trends in crh gene expression data.

Methods

Subjects

Subjects were young adult male Sprague-Dawley rats from Harlan Labs (Indianapolis, Indiana), weighing between 260 and 300 grams at the time of experimentation. The animals were housed in pairs in polycarbonate tubs (47 cm x 23 cm x 20 cm) with access to water and rat chow ad libitum. They were held in four different suites, which were separate from the area where both testing and sacrifice occurred. The housing rooms were maintained on a 12-hour light/dark cycle (lights on at 0700h) and room temperature was held between 20°C and 24°C. Rats were allowed a 2-week acclimation period between arrival and the first testing procedures (surgeries). All care, handling, and use of animals followed ethical guidelines posed by the University of Colorado Institutional Animal Care and Use Committee and adhered to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Experimental Procedure

The experiment used a 2x2 factorial design, the independent variables being the presence of endogenous glucocorticoids and exposure to acute psychological stress (n=6, N=24). Three days before testing, the rats underwent surgical procedures to have their adrenal glands removed, resulting in two glucocorticoid test groups. One half of the rats were bilaterally adrenalectomized via two dorsal lateral incisions under halothane anesthetization, and belonged to the group referred to as the ADX group. The rats that were to remain adrenal-intact still endured a surgery, called a sham surgery, in which all surgical procedures matched that of the ADX group except the adrenals were left in place. The sham surgeries controlled for the stress of surgery. All rats were administered Baytril antibiotic (1.2 mL antibiotic in 10 mL 0.9% saline solution) immediately following surgery. Between surgery and testing, ADX rats were given a
0.9% saline solution as their primary source of drinking water in order to compensate for their inability to retain ions and water as a result of the removal of endogenous aldosterone.

Testing took place 1-3 hours after onset of the light portion of the rats’ photoperiod. Testing began one hour after lights on (0800h) to ensure that all adrenal-intact animals’ CORT levels were at the lowest diurnal levels. Half of the rats (half ADX, half sham) were exposed to a 15-minute acute restraint stress challenge. The rest of the animals (half ADX, half sham) were left in their home cages until sacrifice (HC group).

Restraint Stress Challenge
Rats in the stress challenge cohort were placed in plexiglass cylindrical containers (23.5 cm long, with a diameter of 7 cm, with several air holes) for 15 minutes directly preceding tissue and blood collection. Restrainers prevented major movement, but allowed normal breathing. Restraint stress took place in a room adjacent to, but separate from, the housing suites.

Tissue and blood collection
Rats were taken either directly from their home cages or from the restrainers and immediately decapitated with a guillotine. Animals were sacrificed without anesthesia, as it is a powerful activator of the HPA axis (26). Trunk blood was collected into EDTA-coated (prevents coagulation) test tubes from Becton-Dickinson (Franklin Lakes, New Jersey, USA) and placed on ice. Plasma (containing ACTH) was separated from blood cells by centrifugation for 15 minutes at 4°C, and stored for the ACTH radioimmunoassay in 125-µL aliquots at -80°C.

The fixation method for brain tissue is adapted from the methods described in a paper by Kahn and Watts (2004). Brains were extracted from the skull, blocked in coronal pieces around the hypothalamic sulci between the optic chiasm and mamillary bodies, and placed in glass scintillation vials containing 4% PFA (pH 7.4) for approximately 10 hours at 4°C on a shaker to ensure penetration of PFA. The brain sections were then transferred into scintillation vials containing 30% sucrose dissolved in a 0.1M PBS solution – a solution that enables cells to be in a healthy condition for storage. After 48 hours of shaking in sucrose at 4°C, the brain tissue was taken out of sucrose and frozen in tin foil at -80°C. Brains were cut via cryostat (model 1850; Leica Microsystems, Nussloch, Germany) at -24°C into 25 µm-thick coronal sections and stored
in cryoprotectant (30% sucrose, 30% propylene glycol, 1% polyvinylpyrrolidone in 0.01M PBS buffer, pH7.4) until immunohistochemistry staining.

Radioimmunoassay

ACTH was measured (pg/ml) in duplicate (100 µl plasma) from plasma collected on test day. This single-staged RIA procedure is adapted from a previously established protocol (20,22). 

$^{125}$I radiolabeled ACTH was obtained from Diasorin (Minneapolis, MN, USA); primary ACTH rabbit antiserum (Rb7, final dilution 1:30,000) was donated by Dr. Bill Engeland (University of Minnesota). The assay was sensitive to ACTH concentrations of ~15 pg/ml. The intraassay coefficient of variability was 6%.

Fluorescence Immunohistochemistry

Dual staining of TORC2 and CRH was accomplished by fluorescence IHC. All incubations were performed in 0.01M PBS buffer (pH 7.4) except the final washes, which were done in 0.01M PB buffer (because NaCl is autofluorescent). Following the initial washes, the tissue endured a 30-minute incubation in 0.1% Sodium Borohydride; this step is performed to reduce background fluorescence, however it is harsh on the tissue and reduces its integrity. The initial “block” step utilized a 10% normal goat-serum solution, in addition to 3% dry milk and 1% bovine serum albumin (which reduce non-specific binding), and 0.3% triton X. Primary anti-CRH antibody (guinea pig) was obtained from Penninsula laboratories (catalogue number T-5007) and was diluted to a final concentration of 1:15,000; the secondary antibody for CRH, goat anti-guinea pig Ig, was tagged with Alexafluor 488 (Invitrogen Molecular Probes catalog number A11073) diluted 1:1000. Primary anti-TORC2 antibody (rabbit) was obtained from Calbiochem laboratories (catalog number ST1099) and was diluted to a final concentration of 1:4000; TORC2 secondary antibody was goat anti-rabbit Ig tagged with Alexafluor 594 (Invitrogen Molecular Probes catalog number A11012) diluted 1:1000. Primary antibodies were incubated simultaneously for ~48 hours, shaking at 4°C. Secondary antibodies were also incubated simultaneously for 1 hour at room temperature. After final PB washes, sections were mounted in a 1% glycerol PB solution onto microscope glass plus slides with ProLong Gold antifade reagent with Dapi (Invitrogen Molecular Probes, catalog number P36931).
Image Analysis

Image capture, quantification, and analysis were all done blind to test groups. PVN images were captured and digitized with a Zeiss microscope at 400x magnification. 2-color channel fluorescent (mercury bulb illumination) photomicrographs of at least 4 PVN sections per animal were captured, using Axio-Vision imaging software for both image capture and semiquantitative analysis. Quantification (done blind to test groups) of CRH (green channel) and TORC2 (red channel) utilized the event marker tool to score neurons identified as CRH-immunopositive; neurons were marked as CRH-immunopositive if a clearly identifiable soma was present. It was then determined if each CRH neuron was also TORC2-immunopositive by roughly the same criterion. Each TORC2 positively immunoreactive neuron was then qualitatively characterized as having predominantly cytoplasmic TORC2 versus predominantly nuclear TORC2.

A separate qualitative analysis was performed rating the “brightness” of CRH neurons in the PVN. The analysis was done blind to test groups, and each image was rated on a subjective, 1-5 scale of the brightness of the CRH neurons. All images were captured within a 2-hour time frame, at 400x magnification and camera exposure time was held constant at 200ms for each image.

Statistical Analysis

All statistical tests were performed using the SPSS statistical analysis program 10.5 (Chicago, IL, USA) for Macintosh operating system. The data were first analyzed using two-way ANOVAs to determine whether there was a main effect of either stress or adrenalectomy, or whether there was an interaction between the two variables. If there was a significant F-value in any instance, Fisher’s least significant difference test was conducted to determine pairwise comparisons of interest, \( \alpha = 0.05 \).
Results

ACTH levels reflect trends observed in previous research

Although ACTH patterns in this experimental setting have been well established, data were collected in this study to validate the effectiveness of the acute stress and ADX manipulations on HPA axis activity. The ACTH data were normalized by converting values to a logarithmic scale; after normalization test group differences were as expected. There was a main effect of stress (F_{1,23} = 54.152, p<0.05) and a main ADX effect (F_{1,23} = 102.389, p<0.05). Statistical results were confirmed by Fisher’s least significant difference (LSD) test. This post-hoc analysis also revealed a trend (p=0.065) of higher levels of ACTH in the no-stress ADX group compared to the restraint-stress sham group (Figure 13).

CRH production: negative correlation with endogenous glucocorticoids

As expected, the CRH immunofluorescence for ADX groups was rated significantly brighter than for the sham test groups (ADX effect: F_{1,21} = 85.804, p<0.05; Figure 14). There were no differences in brightness of CRH immunofluorescence between the no-stress and restraint-stress conditions within the sham or adrenalectomy cohorts.

In vivo TORC2 nuclear translocation was not responsive to acute stress or adrenalectomy

The results of the TORC2 analysis did not coincide with expectations in that there were no significant differences between any of the test groups (Figure 15). There was inter-study variability with these results: in the adrenal-intact test group, which is directly comparable to the

Figure 13. ACTH data from main experiment (normalized by logarithmic scale). There was a stress effect in both glucocorticoid cohorts (*,p<0.05). There was also an ADX effect in the stress and no-stress conditions (#,p<0.05). Fisher’s LSD post-hoc test revealed a trend of the no-stress ADX condition having higher ACTH concentrations than the restraint-stress sham condition (&, p=0.065). Original ACTH plasma concentration values (pg/ml) were transformed using a logarithmic scale.

Figure 14. Qualitative (subjective) assessment of CRH peptide "brightness" of immunofluorescence showed an ADX effect (*,P<0.001), but not a restraint-stress effect, with regards to CRH peptide. The analysis revealed an increase in the production of CRH peptide in the ADX cohort.
Discussion

In order to further investigate and expand upon the 2010 *in vitro* tissue culture studies done by Liu et al (2010) and within the Spencer Lab (2010, unpublished) and preliminary *in vivo* studies, we utilized this 2x2 experimental design to observe the possible effects of acute stress and removal of endogenous glucocorticoids upon the activity of TORC2 in CRH neurons. Both of these treatment conditions produce well-documented effects on HPA axis hormone secretion and *crh* gene expression (5,22). However, the testing conditions produced no significant differences in nuclear TORC2 levels between test groups, thereby challenging my hypothesis that acute stress induces increased levels of nuclear TORC2 in CRH neurons of adrenal-intact animals. I also expected to see a slight increase of nuclear TORC2 in the non-stressed ADX group, but the mean level for this group was slightly lower than those of the other groups. Before discussing the TORC2 data, it is important to evaluate the other dependent variables in order to assess the likelihood of accuracy of the TORC2 data.

*ACTH data imply typical HPA axis activity*

The ACTH levels for the various test groups match previously established patterns (5,22). The data indicate that relative HPA axis activity in these animals matched expectations. In the sham group, 15 minutes of acute psychological stress resulted in increased, but regulated, secretion of ACTH. Basal levels of ACTH were elevated in the ADX no-stress condition; the absence of tonic glucocorticoid secretion means that CORT is not able to exert tonic regulation. There was an enormous surge of ACTH with acute stress in ADX animals. This may indicate a
lack of negative glucocorticoid negative feedback within the 15-minute time frame, although feedback would not normally be seen at this time point (Figure 3). Therefore, the unregulated phasic secretion of ACTH in ADX animals may be due to removal of the permissive actions of tonically secreted glucocorticoids (e.g. increased levels of CRH peptide stored in the median eminence causes increased ACTH secretion). Research also indicates that adrenalectomy induces increased sensitivity of CRH neurons at the signal transduction level, which is another interesting possible explanation for the increase in restraint-stress levels of ACTH in adrenalectomized animals (9).

**CRH peptide production increases in the absence of tonic glucocorticoid secretion**

CRH peptide is important to analyze because it can be a reflection of what happens at the level of *crh* gene transcription. There were no differences in the restraint-stress condition within the adrenalectomy cohorts; this is consistent with the notion that new CRH peptide is not manufactured within fifteen minutes of the onset of stress. However, in the absence of tonic glucocorticoids, CRH peptide becomes more concentrated in the neurons, as assessed by a significantly higher rating in brightness of immunofluorescence between sham and adrenalectomy conditions. This supports that there was an upregulation in *crh* gene expression in the absence of glucocorticoids (Figure 16). It is plausible that CRH concentrations were higher in the median eminence (the axon terminals of CRH neurons) as well. If secretion of CRH peptide is higher at the median eminence, this may be the cause for the increase in both basal and phasic increases in ACTH in the absence of endogenous glucocorticoids. If this is the case, then at least one level of regulation occurs at the level of the CRH neuron (27).

**TORC2 translocation is not sensitive to either tonic or phasic secretion of glucocorticoids**

The results obtained from this study do not correspond with what we had expected to see. The data for the sham cohort deviate from those of prior studies in that there was no difference
between the cellular location of TORC2 in CRH neurons of unstressed and stressed animals. Because *crh* gene transcription has been shown to peak at 15 minutes of restraint stress (5), in theory TORC2 activity should increase in response to restraint-stress in both the sham and ADX conditions, which was not observed in this study. If the data from this experiment show the true nature of TORC2 activity in the PVN, then it can be concluded that TORC2 is not responsive to 15 minutes of restraint stress *in vivo*.

The ACTH data are consistent with normal HPA activity indicating cellular activation of CRH neurons to some extent. This, coupled with an increase in CRH peptide production in the absence of glucocorticoids, shows that there are both genomic and regulatory changes in the HPA axis in response to acute psychological stress and tonic secretion of glucocorticoids. There was no difference between nuclear TORC2 levels for either group within the ADX cohort, indicating that the removal of tonic glucocorticoid secretion does not have an effect on TORC2 activity in CRH neurons. The preliminary *in vitro* study showed a similar trend in that adding CORT to the culture did not produce differences in TORC2 activation, as compared with vehicle (Figure 9). However this method may be more applicable to the impacts of phasic glucocorticoid secretion, the effects of which are probably not yet evident with 15 minutes of restraint stress. The combination of these data sets strongly indicate that tonic secretion of glucocorticoids do not influence TORC2 activity. However, the fact that there were no differences in restraint stress for either the sham or adrenalectomy cohorts was surprising, as this is inconsistent with previous findings (Figure 12).

Liu et. al published a study in 2011 which investigated TORC2 activity in the PVN *in vivo* with an experimental design similar to that of this project, however Liu et. al used dramatically different restraint-stress time points for their measures. In this study by Liu et. al rats were restrained for one hour, removed from stress, and then sacrificed either 30 or 240 minutes after being taken out of restrainers and returned to their home cage. The data showed robust nuclear TORC2 percentages in the dorsolateral PVN (using DAB immunohistochemistry) at 30 minutes, but not at 240 minutes (p<0.01). The study also utilized chromatin immunoprecipitation assays for TORC2 antibodies, and found an increase in TORC2 binding to *crh* promoter DNA at 30 minutes, but not 240 minutes (p<0.01) (17). This shows that there is TORC2 activity *in vivo* in response to restraint stress, but perhaps on a different time scale than what was measured in the project for this paper. However, it is still difficult to relate this to the fact that activation of the
PKA pathway is required for both TORC2 activation and crh promoter activity. Cellular stimulation occurs instantaneously (within seconds) of the perception of stress, and is inhibited at 30 minutes of restraint stress as shown by a reduction of c-fos mRNA at this time point (5), yet Liu et. al showed both TORC2 and crh gene activity 90 minutes after the onset of stress. Liu et. al (2011) did not give an explanation for why both would occur 30 minutes after the removal of restraint stress (90 minutes after the onset of stress). It is important to note, though, that the highest average percentage of nuclear TORC2 Liu et. al observed was only about 60% (17).

With regards to the data obtained from this study, it is also possible that there were other important variables that have not been considered, one of which could be glucocorticoid negative feedback influence on the cellular location of TORC2. Crh gene expression returns to normal levels after approximately 30 minutes of acute restraint stress (Spencer Lab, unpublished data 2009), and the rapid return of crh gene expression to basal levels may be due to glucocorticoid negative feedback. At fifteen minutes after the onset of stress (as it may be – glucocorticoids could be synthesized, released, and in the brain by this time), it is possible that glucocorticoids play a role in exporting TORC2 out of the nucleus, thereby terminating crh gene transcription. This would not contradict crh gene transcription data – even if actual transcription had ended by 15 minutes of restraint stress (which is when crh gene expression peaks), there could still be previously synthesized hnRNA in the nucleus. If this were the case, then levels of nuclear TORC2 should be higher prior to 15 minutes of restraint stress, which has not been indicated thus far. However, both the first and the third preliminary studies showed that TORC2 was highly nuclear in hypothalamic neurons 30 minutes after administration of forskolin or restraint stress, respectively (Figure 9, Figure 12), makes this explanation unlikely. In addition, a widely accepted mechanism for feedback is that occupied glucocorticoid receptors prevent further transcription of crh gene by binding directly to the DNA at an nGRE (11), making it unlikely that crh gene transcription is directly regulated by TORC2 being exported from the nucleus.

While these proposals are worthy of consideration, there was also a high degree of intra-group variability in this experiment, and it is important to appreciate the differences seen in individual animals within test groups. The ranges for the data are as follows: sham/NS = 9.64-40.0%, sham/RS = 16.1-47.0%, ADX/NS = 4.6-31.3%, and ADX/RS = 9.6-53.6% nuclear TORC2 in CRH neurons. There are obvious differences in nuclear TORC2 between individuals within the
same test group, as exemplified by Figure 17. Because there was a high degree of variability within test groups, the validity of these results may be questionable, therefore it was important to investigate this possibility. The differences could be attributed to individual genetic differences of the animals, and could be manifested in either the individual animal’s interpretation of the stressful event or the time scale of various intracellular processes. TORC2 values were compared with ACTH concentrations (which indicate the degree HPA axis activity) using the Pearson correlation coefficient test, which revealed no significant correlation. It may also be viable that TORC2 is responsive to some other, unknown variable that has not been identified.

Sources of Error

Inter-study variability must also be considered, and these differing effects may be explained by experimental error within either the pilot or the main study. If the error lies within the pilot study, it is likely related to the size of the test groups, as they were rather small (n=4). However, the statistical analysis indicated that there is a reasonable probability that the differences were not due to chance (P=0.067). An additional source of error, which may be the case for both studies, is error within TORC2 cytoplasmic versus nuclear TORC2 characterization. This analysis may produce error because it is subjective in nature. However, the method for characterization has been practiced and perfected through many analyses, and cannot definitively be responsible for variability in the experiment. Another source of error may be that the animals that were supposedly from a “no-stress” condition. The animals could have had HPA axis activation upon being carried from their home cage to the decapitation platform, or from the actual process of decapitation; however ACTH data did not indicate this was the case, as there was relatively low variability within the no-stress groups for both the sham and ADX cohorts.
Another weakness of this experiment is that data reflecting \textit{crh} gene transcription was not collected. The way that tissue is preserved to enhance immunohistochemistry staining, which is 10-hour post-fixation followed by storage in cryoprotectant, makes it virtually impossible to collect data about RNA levels. Although it is still useful to compare averages for \textit{crh} hnRNA levels of other studies, it would have been useful to have data points from individual animals from this study in order to explore if there is a correlation between \textit{crh} gene expression and TORC2 activity.

Although error may contribute to the negative results of the study, it is also important to consider the nature of the TORC2 protein observed up to this point. In no-stress conditions, the average amount of TORC2 found within the nucleus of CRH neurons hovers at around 15%, and the average for the stress condition in studies showing significant differences in percent nuclear TORC2 is only at about 30%, with the highest percentages at around 50%. If TORC2 is a coactivator of pCREB and is required for \textit{crh} gene transcription as suggested by Liu et. al (2009, 2010), then levels of stress-induced TORC2 nuclear translocation seem surprisingly low, even in the stress condition when \textit{crh} gene transcription peaks (5). HPA axis activity and CRH peptide upregulation both indicate that there were differences in hormonal and genomic action, but TORC2 appeared largely unresponsive to any of the testing conditions. This may be an indicator that TORC2 is not responsive to stress or glucocorticoids \textit{in vivo}, however it is apparent that further investigation is necessary.

\textit{Future directions}

Because activation of PKA by forskolin potently induced translocation of TORC2 to the nucleus \textit{in vitro} but this movement was not observed \textit{in vivo}, it is safe to assume that there are other factors influencing TORC2 activity in the PVN. An interesting study may be to insert a guide cannula directly into the PVN and administer either vehicle or forskolin, and measure TORC2 translocation under both stressed and non-stressed conditions. It would also be interesting to infuse vehicle or forskolin in a chronic manner, to observe if there are long-term differences in hormonal (ACTH and CORT) activity in response to an increase in cellular activity of the CRH neuron.

A finding from the second preliminary study that has not been addressed is the highly significant increase in nuclear TORC2 at different times of day. In this small study, there was a
high percentage of nuclear TORC2 in brains harvested after 1400h, which may indicate a relationship of TORC2 activity to circadian rhythms. This is also supported by increases in both crh and c-fos gene expression, as shown by Girotti et. al (Figure 6, Figure 7). It would be interesting to examine the activity of TORC2 over a time course throughout the day to see if there are diurnal differences in its cellular location.

Also, because Liu et. al (2011) saw a significant increase in CRH neurons with nuclear TORC2 at a much later time point than was studied in this project, it would be useful to have a restraint-stress time course at time points between 0 and 240 minutes. An interesting analysis for this (and other) suggested study would be to investigate TORC2 activity in other regions of the brain, such as the hippocampus and amygdala where TORC2 is also found.

Finally, because TORC2 location characterization has proven to be difficult, it may be useful to explore better methods of staining and analysis. One of these methods might be examination of colocalization of TORC2, CRH, and DAPI, which is a DNA stain that marks the nucleus of all cells. A second method for improving TORC2 characterization would be to use confocal microscopy, which provides a higher-resolution image of individual planes of the tissue slices, making identification of cellular locations more straightforward. This would use fluorescence IHC, and overlapping images taken of these different molecular signals may assist in characterization of TORC2 location. It would also be useful to establish a method of tissue fixation in which both immunohistochemistry as well as in situ hybridization can be used, which would allow TORC2 and crh gene expression to be measured in the same animal.

**Conclusion**

In this study, TORC2 was shown to be unresponsive to acute psychological stress as well as to the removal of tonic secretion of glucocorticoids. This differed from expectations, either due to experimental error, or is a true reflection of what happens in vivo under these experimental conditions. It is clear through in vitro studies that TORC2 is responsive to stimulation of the PKA pathway, and the recent in vivo study by Liu et. al (2011) indicates that TORC2 activation can be observed at a later time point. In addition, strong evidence for TORC2’s contribution to crh gene expression in vitro is persuasive enough to imply that TORC2 activity in the hypothalamus induces crh gene expression in vivo. Studies conducted up to this point still strongly suggest that TORC2 might be responsive to restraint stress, and does deserve further investigation.
References


