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Localization of Extracellular Regulated and Calcium-dependent Calmodulin Protein Kinases in Posterior Hypothalamic Neurons Displaying Stress-induced Fos Induction

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Localization of Extracellular Regulated and Calcium-dependent Calmodulin Protein Kinases in Posterior Hypothalamic Neurons Displaying Stress-induced Fos Induction

A thesis submitted in partial fulfillment of the requirements for the
Degree of Bachelor of Arts with Honors in Neuroscience at:

University of Colorado at Boulder

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Abstract:

Historically, stress habituation research has largely focused on the reduction of the hypothalamic-pituitary-adrenal axis response to repeated stress exposures. The habituation of the HPA axis to prolonged stress is caused, in part, by negative feedback mechanisms, the activation of extensive stress-related neural circuitries, but also more complex enzymatic cascades underlying associative memory mechanisms. The results of previous investigations have implicated the MAP Kinase and CaM Kinase pathways as potential signal transduction pathways activated in stress adaptation. In addition, the amygdala and the hippocampus are well-known brain areas for learning and memory, however a recent study has shown that the posterior hypothalamus may also be a critical site for simple forms of learning like habituation. Building on this research, this project seeks to determine if microtubule associated protein kinases 1/2 (ERK) and calcium-dependent calmodulin kinase II alpha (CaMKII α) are in cells characterized as active by stress-induced c-Fos protein expression in the posterior hypothalamic (PH) nucleus. In the following series of studies, we demonstrate with immunohistochemistry that ERK and CaMKII α , but not Elk-1 can be localized in stress-responsive neurons of the posterior hypothalamus. Immunohistochemistry was used to measure the functional activation of ERK by assessing its level of phosphorylation (pERK) and the presence of CaMKII, in the posterior hypothalamus of acutely stressed rats. The phosphorylation of ERK is time-dependent; whereas basal expression was observed in control animals, a significant increase was observed in rats exposed to 15-30 minutes of stress. It has been shown that Fos protein is expressed in active pathways in the brain and we found that pERK but not CaMKII is significantly co-expressed in the same cells with Fos. These findings suggest that ERK is present in neurons that are likely signaling during stressful exposures, and could be involved in long-term plastic changes underlying, for example, habituation to repeated stress.

Acknowledgements

I would like to express my greatest gratitude to the people who have supported me throughout this project, beginning with the members of the Campeau Lab who have taught me the basics of neuroscientific research, lifted the monotony of work in the dungeon and who are happy to see me succeed now. It was an honor working with them.

This work could not have been accomplished without the social support of my family and closest friends. I am lucky to have a wonderful family who will support me no matter what. Their accomplishments and inexhaustible encouragement has shaped my strong-willed personality that was absolutely necessary in the completion of this project. As for my friends, they have been there at every step along the way. I will cherish the experiences we shared and will share together in the future.

I would like to extend my gratitude to the members of my thesis committee, for their constructive criticism, love of science and academia and time devoted to hearing this finished project.

Most of all I would like to thank my mentor and principal investigator of the Campeau Lab, Dr Serge Campeau, who gave me the golden opportunity to become an undergraduate researcher as a sophomore and has helped me receive three research scholarships over the years. Without his tremendous guidance, insight and invaluable motivation in times of distraught due to fruitless research, this project would not have been possible.

Contents

Abstract	i
Acknowledgements	ii
1. INTRODUCTION	1
1.1 The phenomenon of habituation.....	1
1.2 Posterior Hypothalamus.....	2
1.3 Stress habituation and synaptic plasticity – LTP & LTD.....	3
1.4 Role of CaMKIIalpha, pERK & pElk-1.....	5
1.4.1 CREB.....	6
1.4.2 CaM Kinase.....	7
1.4.3 phospho- ERK.....	9
1.4.4 phospho-Elk-1.....	11
1.5 Fos as a marker of activated neurons.....	12
2. MATERIALS AND METHODS	13
3. RESULTS	17
4. DISCUSSION / FUTURE DIRECTIONS	20
5. REFERENCES	25

1. Introduction

1.1 The Phenomenon of Stress Habituation

Habituation is a widespread phenomenon observed in most species and is defined as a reduction in response amplitude to a stimulus upon repeated exposures to the same stimulus. While habituation is considered the simplest form of learning and is exhibited ubiquitously in vertebrates and invertebrates, the underlying cellular mechanisms mediating this process is yet to be fully understood (Esdin, Pearce & Glanzman, 2010). Habituation is established from most classes of stimuli, including stressors like noise exposure or restraint. These stressors also elicit habituation whereby reduced stress-elicited responses are observed following repeated stressor exposures (Grissom & Bhatnagar, 2008).

There exist several measures of stress elicited responses in rats which can be observed following habituation to stress. Among these measures are the release of the hypothalamo-pituitary-adrenocortical axis (HPA) hormones adrenocorticotropic hormone (ACTH) and cortisol (or corticosterone in rodents), the adrenomedullary hormones epinephrine and norepinephrine, various autonomic responses such as tachycardia, blood pressure, hyperthermic core body responses, and several behavioral responses.

The neuronal circuitry associated with psychological stress responses involves the limbic system. Psychological stressors first activate the limbic system which then projects to several subcortical areas such as the Preoptic Area (POA), the dorsomedial hypothalamus (DMH) and the bed nucleus of the stria terminalis (BNST) through which sensory information is relayed to the medial parvocellular paraventricular nucleus of the hypothalamus (mpPVN) to initiate HPA axis response to stress. The limbic system is a system of interconnected nuclei such as the amygdala, hypothalamus and hippocampus which are critical for motivated behaviors, emotions

and long-term memory. Of these limbic-associated structures, the hypothalamus is an important effector node for responses regulated by the limbic system (see Figure 1). Responses to psychological stressors can be innate like a rat's fear of predators (cats, ferrets, etc.), or alternatively they can be conditioned, examples of which include the classical conditioning of weak visual stimuli and acoustic stimuli paired with electrical shocks. One brain region responsible for the formation of contextual memory in response to both innate and conditioned stressors is the amygdala. When activated by an environmental stressor, the amygdala will send signals to several subcortical relay nuclei.

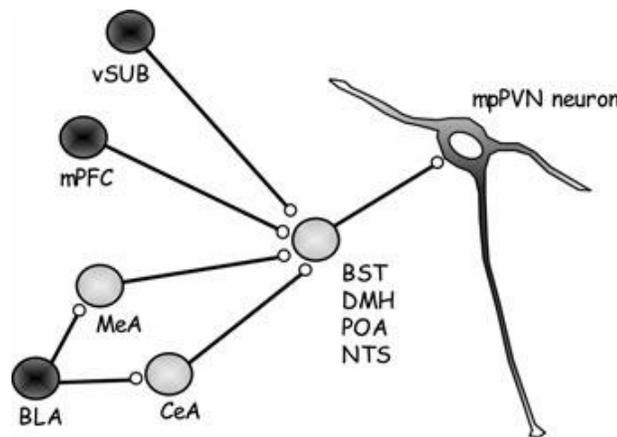


Fig.1 The ventral subiculum and medial prefrontal cortex excite the BNST/DMH/POA through glutamatergic projections upon GABAergic neurons of the BNST, which in turn inhibit the mpPVN through activation of GABAergic neurons. The basolateral amygdala excites the medial and central amygdala which excite the mpPVN through disinhibition of the BST, DMH and POA (Jankord & Herman, 2008).

1.2 The posterior hypothalamus as a region important in stress habituation

It has been shown that inactivation of the posterior hypothalamic nucleus/dorsomedial hypothalamus region with the GABA_A receptor agonist muscimol reduces many acute stress-elicited responses when injected immediately prior to stress (Nyhuis, Day & Campeau, 2012).

Muscimol also disrupts stress habituation when injected prior to repeated stressor exposures. In addition, vehicle injected animals repeatedly exposed to loud noise showed corticosterone habituation which was attenuated following repeated injections of muscimol prior to each stress exposure (Nyhuis, Day & Campeau, 2012).

The rostral Raphe Pallidus (rRPa) and the paraventricular hypothalamic nucleus (PVN) have previously been shown to be activated during acute stress. The posterior hypothalamic region of acutely stressed rats previously injected with retrograde tracers in the rRPa and/or the PVN displayed the highest levels of co-localization with the immediate early gene protein product Fos. This indicates that the posterior hypothalamic nucleus projects to these two areas, further supporting its involvement in acute responses to stress. The posterior hypothalamus also receives sensory information from the auditory thalamus creating a connection between auditory processing and a region perhaps critical in mediating stress responses (Campeau & Watson, 2000). Combined with additional anatomical evidence indicating that the posterior hypothalamic nucleus is innervated by several sensory regions, and in turn projects to many brain regions that regulate neuroendocrine and autonomic responses elicited by stress, this region is ideally situated to modify its input-output relationship through synaptic modifications and provide the basis for habituation to stress (Nyhuis, Day & Campeau, 2011).

1.3 Stress Habituation and Synaptic Plasticity – LTP & LTD as possible mechanisms

Adaptation to stress at the cellular level involves the process of synaptic plasticity, which is the strengthening or weakening of a synapse between two neurons due to use or disuse. The flexibility of synapses to undergo morphological and functional changes is central to understanding learning and memory. Synaptic strengthening occurs via an extension of dendritic spines, the insertion of excitatory receptors and/or activation of silent synapses of the post-

synaptic cell, magnifying signaling between connected neurons. The weakening of the synapse happens through the internalization of receptors from the membrane and the retraction of dendritic spines.

The leading candidate mechanisms of synaptic modifications associated with a variety of learning and memory processes include long-term potentiation (LTP) and long-term depression (LTD) (Malenka & Bear, 2004). Preliminary results indicate that posterior hypothalamic nucleus neurons projecting to the autonomic nucleus controlling tachycardia and hyperthermia (rRPa) mostly contain glutamate as their neurotransmitters (H. Day & S. Campeau, unpublished results). However, several additional neurons of the posterior hypothalamus contain the neurotransmitter GABA. Conceivably, LTP- or LTD-like mechanisms could mediate the development of response reductions upon glutamate or GABA-containing neurons at the level of the posterior hypothalamic nucleus.

Several intracellular signaling cascades and their associated enzymes have been reported to mediate synaptic plasticity via LTP or LTD-like mechanisms. One of the effects of LTP in several brain regions is the phosphorylation, increased insertion and increased average current mediated through α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) subtype of excitatory amino acid receptors. Once the post-synaptic membrane is depolarized through AMPA receptors in response to a stimulus, magnesium-gated NMDA receptors can be activated to further depolarize post-synaptic elements and allow influx of calcium, which serves as a second messenger associated with the strengthening or weakening of synapses. This effect is mediated through a signaling cascade involving the phosphorylation/activation of several enzymes, including calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase A (PKA) or protein kinase C (PKC), eventually leading to the phosphorylation of the transcription

factor cAMP-response element binding protein (CREB), which is necessary for the effects observed on post-synaptic AMPA receptors (Kennedy, Beale, Carlisle, & Washburn, 2005; Malenka & Bear, 2004). Interestingly, LTD has been associated with several *dephosphorylating* processes, including AMPA receptor subunits and CREB, in addition to the phosphorylation of the transcription factor Elk-1 (Thiels, Kanterewicz, Norman, Trzaskos, & Klann, 2002). The removal of AMPA receptors from the membrane and the consequent reduction in the amplitude of post-synaptic depolarization leading to decreased signal transduction, provides a probable mechanism explaining the response reduction associated with adaptation to stress, however this explanation, as of now, is still a theory.

As mentioned above, there are several protein kinases and transcription factors that play a part in the phosphorylation and dephosphorylation of AMPA receptors enhancing plastic changes. If these kinases play a role in habituation, they would be expected at the very least to be present in posterior hypothalamic neurons, and display some increases in functional activity following a stressor, and decrease in functional activity with repeated exposure to that stimulus. In an effort to further understand the plastic processes underlying the development of stress habituation, the localization of kinase enzymes previously associated with a variety of plastic processes at the level of the posterior hypothalamic nucleus was investigated.

1.4 Role of CREB, CAMKII, pERK and Elk-1 in Synaptic Plasticity

Multiple signal transduction pathways have been implicated in mediating habituation, learning and memory formation including calcium/calmodulin dependent kinases, mitogen activated protein kinases (MAPK), protein kinase A, and protein kinase C, among others. These pathways converge on the transcription factor CREB which, when active, is conducive to plastic changes (Barco, Jancic & Kandel, 2008). There is an extraordinary level of interaction and

convergence between these transduction pathways, increasing the complexity of synaptic plasticity. Due to this overlap, events in one pathway can activate enzymes in another, leading to the simultaneous induction and propagation of these signaling cascades. Upon reflection, a fine integration of these pathways is and should be necessary for functional alterations of neurons. Due to this complexity, we aim to focus on the first two pathways, the CaMK and MAPK signaling pathways (Sweatt, 2004).

1.4.1 CREB

In order to understand how cAMP-dependent synaptic plasticity by the MAPK and CaMK pathways occurs, it is important to illustrate the role of CREB in this process. There are several signal transduction pathways that converge on the phosphorylation of CREB, the two of interest here are the CaMK and MAPK pathways (see Figure 2). While the interaction between CREB and CaMKII remains largely unresolved, evidence shows that increased levels of activated CREB (phosphorylated or phospho-CREB) and CaMKII are the causes of the generation of silent synapses in plasticity. Through the regulation of CaMKII, CREB can augment AMPA receptor density in the synapse (Marie, Morishita, Yu, Calakos, & Malenka, 2005). Aplysia phosphoCREB (ApCREB) controls the transcription of several immediate-early response genes that play a role in habituation, such as the expression of ubiquitin hydrolase in Aplysia which regulates the regulatory domain of PKA, known to constrain long term facilitation. CREB also controls the production and translocation of mRNA critical for the generation of new synaptic connections branching from the synapse that initially received stimulation. Therefore, gene expression regulated by phospho-CREB contributes to the growth of new synapses and the stabilization of existing synaptic connections (Barco, Jancic & Kandel, 2008).

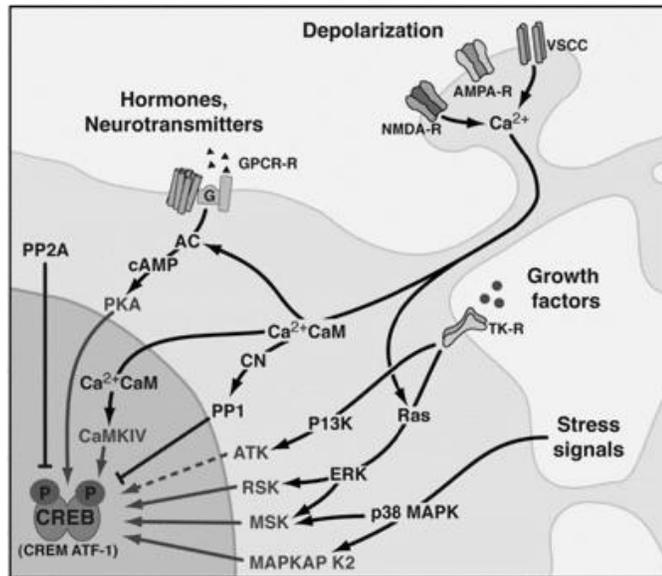


Fig 2: External stimuli, such as stress, regulates the phosphorylation of CREB via converging protein-kinase pathways.

1.4.2 CaM Kinase

CaMK is part of a G α Q-G protein coupled receptor signaling pathway where upon the binding of a ligand to a metabotropic receptor, a conformational change occurs in the associated Gq protein, causing a guanosine diphosphate to be exchanged for a guanosine triphosphate and causing the alpha subunit (GTP coupled) to dissociate from the $\beta\gamma$ subunits. The GTP-Gq complex then binds to an inactive membrane bound phospholipase C, activating it and causing the cleavage of Phosphatidylinositol diphosphate (PIP₂) into Inositol triphosphate (IP₃) and diacylglycerol (DAG). IP₃ is released into the cytosol where it triggers Ca²⁺ release from the endoplasmic reticulum, among others, and DAG remains in the membrane where it activates protein kinase C (PKC). After intracellular calcium levels rise, calmodulin binds four calcium ions and changes its conformation to form an active calcium /calmodulin complex. The two globular ends of the complex attach to the inhibitory domain of a target protein, in this case CaMKII (see Figure 2). Upon Calmodulin binding, the kinase engages in autophosphorylation which perpetuates its activity even after calcium levels drop. Once phosphorylated/activated,

CaM Kinase will catalyze the phosphorylation of substrate proteins by transferring phosphate groups from adenosine triphosphate (ATP) to the hydroxyl (OH) groups of serine and threonine residues of those substrate proteins. The addition of an electrically charged phosphate group induces a conformational change and subsequently the biological activity of the target proteins (Nestler, Hyman & Malenka, 2009).

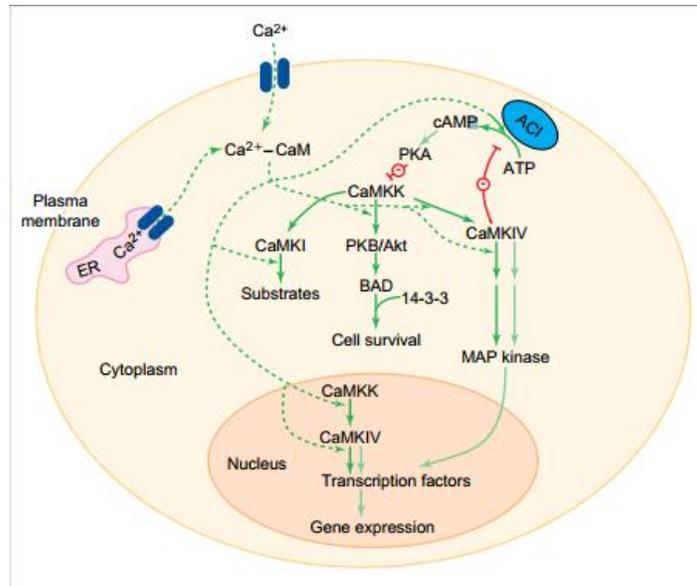


Fig 3: Ca^{2+} - Calmodulin dependent Protein Kinase Signaling Pathway (Soderling, 1999)

The CaM Kinase pathway is suspected to underlie neuronal plasticity associated with learning and memory through the activation and deactivation of CREB by CaMKII and calcineurin (protein phosphatase 2B), respectively (Barco, Jancic & Kandel, 2008), as well as through interaction with AMPA receptors. Tetrameric AMPA receptors are comprised of four different subunits, out of which, the phosphorylation or dephosphorylation of GluR1 and GluR2 have been linked to LTP and LTD. Upon LTP induction, CaMKII attached to membrane-bound NMDA receptors is known to recruit a protein, SAP97, member of a membrane-associated guanylate kinase (MAGUK) family protein. SAP97 has been shown to interact with the C-terminal tails of the GluR1 subunit, facilitating the insertion of cytosolic AMPA receptors into

the postsynaptic density. The addition of AMPA receptors into the synapse, as mentioned before, contribute to the strengthening of synaptic communication and dendritic arborization. Due to this effect, CaM Kinase is an attractive candidate upon which LTP/LTD depends (Lee, 2006). Here, we aim to investigate whether CaMKII was expressed especially in posterior hypothalamic neurons that also display stress-induced increases in the immediate early gene c-fos, to provide evidence for its putative role in the plasticity underlying habituation to repeated stress exposures. To do this, immunohistochemical detection of CaMKII was performed following an acute exposure to loud noise stress.

1.4.3 MAP Kinase

The mitogen activated protein kinase (MAPK) pathway has polymodal activation. Calcium can directly trigger the activation of Protein Kinase C which will activate Rat Sarcoma (RAS) protein or indirectly trigger Protein Kinase A activating Ras related protein 1 (Rap 1). Ras can also be activated through neurotropic factors binding to receptor tyrosine kinases. Once these 2nd and 3rd messengers have been activated, Ras will phosphorylate Raf-1 and Rap 1, which will phosphorylate B-Raf, eventually leading to the phosphorylation and activation of MEK and in turn ERK (see Figure 3). Finally the kinase cascade leads to activation of Activation protein 1 (AP-1) family of transcription factors. The best known AP-1 are Jun and FOS, which heterodimerize and move to the nucleus to effect transcriptional changes (Nestler, Hyman & Malenka, 2009).

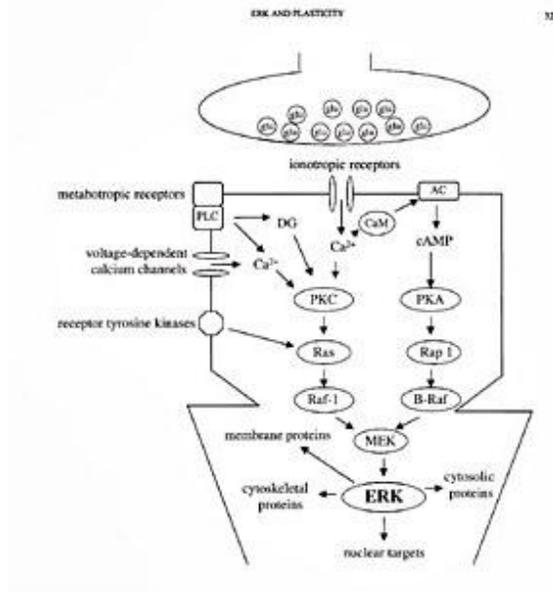


Fig 4: Mitogen Activated Protein Kinase Pathway (Thiels & Klann, 2001)

Learning and memory deficits from Ras/ERK hyper activation in a mouse model of neurofibromatosis demonstrate that healthy functioning of ERK is necessary for synaptic plasticity associated with learning (Sweatt, 2004). The activation of the ERK signaling pathway via Acetylcholine activated hippocampal muscarinic receptors show that without sufficient ERK phosphorylation, the induction of LTP does not happen. In conjunction with evidence that ERK blockers impede the progression of LTP, it may be concluded that ERK is critical in the induction of LTP (Giovannini, 2006). Since LTP is one of the candidate mechanisms underlying adaptation to stress, ERK expression in the PH could mediate long-term synaptic plasticity.

A rise in post-synaptic levels, the translocation to the nucleus and phosphorylation of apCREB-2 by pERK, implicated this kinase in long term facilitation (LTF). These changes were observed in the gill-withdrawal reflex in *Aplysia* as well as in sensory cell cultures subjected to repeated 5-HT applications, previously shown to induce LTF (Michael, Martin, Seger, Ning, Baston & Kandel, 1998). Long term facilitation (usually used in reference to *Aplysia*) and Long term potentiation (used in reference to mammals) are phenomenologically the same in that they

both increase synaptic efficacy. Evidence that transcriptional regulators and a cell adhesion molecule (apCAM) both contain consensus phosphorylation sites for ERK, supports the role of ERK in synaptic plasticity in habituation. These experiments combined with the finding that LTF is blocked by a MEK antagonist, directly responsible for the activation of ERK, further elucidate the possible causal relationship between ERK and plasticity (Thiels & Klann, 2001). Observed increases in ERK2 mRNA levels after the establishment of NMDA receptor-dependent LTD and the subsequent CREB-dependent transcription seemingly provide a link between ERK cascades and CREB-dependent transcription. Similarly, elevated expression of B-Raf and Raf-1 (found upstream in the ERK activation cascade) after LTP induction in dentate gyrus cells demonstrate consistently that ERK is mediating post-LTP gene expression. Therefore, based on these findings, our second aim is to detect the presence of the active form of ERK, phospho-ERK (pERK) in the posterior hypothalamus, especially in neurons also expressing stress-induced Fos protein elevations.

1.4.4 p-ELK-1 upregulated in LTD

Elk-1 is a transcriptional activator that is responsible for the transcription of c-Fos. Elk-1 seems to be a downstream target of ERK in the MAPK pathway (Tian & Karin, 1999). Elk-1 has a transcriptional binding domain on the C terminal tail conserved with a consensus sequence for MAP Kinases (Cruzalegui, Cano & Treisman, 1999). Findings demonstrating the occurrence of synaptic plasticity via Elk-1/CREB interaction following traumatic brain injury, suggest that ERK may be responsible for transcriptional changes induced by CREB through Elk-1 (Hu, Liu, Bramlett, Sick, Alonso, Chen & Dietrich, 2004). From these findings, we hypothesize that stress habituation might be associated with Elk-1 activation, and propose to look for the expression of

the activated form of this protein, phospho-Elk-1 (pElk-1) in posterior hypothalamic neurons also expressing stress-evoked Fos induction.

1.5 Fos as a marker of activated neurons

Responses to acute stress exposure at the cellular level can be measured by looking at levels of the transcription factor Fos. c-Fos is an immediate-early gene (IEG) rendering this transcription factor a marker for activated neurons. An IEG is a gene that responds to a wide variety of stimuli, before the synthesis of new proteins. It has a low level of expression under basal conditions and rapid induction in response to extracellular signals. c-fos mRNA is detectable a few minutes after transcriptional stimulation, peaks within 30-60 minutes and diminishes after 2-3 hours. C-Fos gene expression results from the quick phosphorylation of CREB (cAMP response element binding protein) via protein kinase A and CaM kinase. Due to the low basal level of c-Fos expression, but its sizable induction by a variety of stimuli, including various stressors, c-fos has been employed extensively as a tool for specifying neuronal pathways displaying increased activity in the behavioral and neuroendocrine responses induced by stress. Brain regions observed to express c-fos mRNA in response to stimuli exposure includes the lateral septum and anterior BNST among others (Kovacs, 1998). Since ERK and CaMKII have been closely associated with a variety of plastic processes, and because posterior hypothalamic neurons appear necessary in the mediation of habituation to stress, the current study was designed to determine if ERK and CaMKII are possible transduction systems present in cells of the PH that are known to be active through Fos with loud noise stress.

2. Methods

Behavioral Procedure: Male Sprague–Dawley rats (n=14) were housed four to a cage in a colony facility and provided food and water. The afternoon before the experiment, the animals were transferred to individual cages and taken to the testing room and placed in noise boxes so they can adjust to the novel environment. This was necessary so molecular, hormonal and cardiovascular changes in response to stress would solely be attributable to the stressor and neither to the novel environment nor to transportation. Each acoustic chamber was ventilated and double wooden with a red fluorescent light. The intensity level was set to 100 decibels (A scale) and the exposure time was 30 minutes for the experimental group on the morning of the experiment. After the control, 15, or 30 minute loud noise exposure, the animals remained in the chambers for an additional 15 minutes. The control group stayed in the chamber for the same duration of time. This duration of exposure and post-stress interval was chosen to maximize the detection of loud noise-induced ERK phosphorylation, which is relatively quick in response to stress exposure, and the detection of loud noise induced Fos, which requires a longer elapsed time for expression (Masini, Babb, Nyhuis, Day & Campeau, 2012).

Tissue processing and sectioning: For the first experiment, the animals (n=4) were anesthetized with sodium pentobarbital (Fatal Plus) and transcardially perfused. For the second (n=6) and third experiment (n=4): following the noise or control exposures, the animals were decapitated, their brains rapidly removed, blocked on ice and placed in vials to be soaked in fixative solutions. We used this alternate fixation procedure to prepare tissue instead of the traditional aortic perfusions because our previous experiments yielded very strong expression of phospho-ERK (pERK) even in the control rats, likely due to the lengthy perfusion manipulations. Therefore, to obtain more consistent results and lower basal values, a faster tissue isolation

method was used. The brains were immersed in sodium acetate-buffered *para*formaldehyde (4% PFA/0.1M Sodium Acetate, pH=6.5) and chilled for ~6-8 hours at 4° C, rotating on a platform. This pH provides an ideal permeation rate for the PFA into the brain tissue (Khan & Watts, 2004). After 8 hours, the brains were removed from this initial solution, blotted dry and transferred to a solution of Sodium Borate buffered PFA (8% PFA/.2M Na Borate, pH=9.5) and soaked for 4 days at 4°C, shaking. Next, the fixative was transferred to a Sodium phosphate buffered glycerol solution (0.1M Na phosphate/20% glycerol solution, pH=7.4) at 4° C, shaking, for 2 days until brains were completely saturated and had no buoyancy. The brains were then flash frozen in -30°C isopentanes and wrapped in foil and stored in -80°C freezer until sectioning. Sectioning was performed using a cryostat (Leica 1450). The brain blocks were mounted using M1 and cut into 30-micrometer coronal sections with no roll plate at -28°C. The sections were collected into 6-well plates filled with cryoprotectant solution.

Immunohistochemistry: In order to determine the titration of antibody that should be used to label cells, immunohistochemistry was performed on sections of the brains of acutely stressed rats (n=4). The brain sections used were chosen mostly from the hypothalamic paraventricular nucleus (PVN), the bed nucleus of the stria terminalis (BNST) and lateral septum since these areas have previously been shown to display pERK, CaMKII and Fos induction in response to stress. On day 1, the tissue was washed in 6x5' in 1xPBS (.1M (10x) PBS: 80g NaCl, 2g KCl, 2g KH₂PO₄, 11.5g Na₂HPO₄·7H₂O dissolved in 1L of Milli-Q H₂O, pH=7.4). Then, it was incubated in 0.3% Hydrogen Peroxide for 15 minutes at room temperature, shaking on a rotating plate, followed by another 6x5' wash in 1xPBS. Next, it was incubated in Avidin blocking reagent for 20 minutes at room temperature, shaking, followed by 1x5' in 1xPBS wash. After, the tissue was incubated in Biotin blocking reagent (Avidin/Biotin blocking reagent kit;

Vector Laboratories Inc., Burlingame, CA) for 20 minutes in a similar manner to the Avidin, followed by 1x5' in 1xPBS wash. Then, the tissue was pre-incubated in a buffer containing 1% bovine serum albumin, 0.5% Triton-X 100, 0.25% Carrageenan-Lambda, all dissolved in .01M PBS (immune buffer), for 1 hour at room temperature. As the last step for this first processing day, the sections were transferred into the primary antibody that has been appropriately diluted in immuno buffer and allowed to react for 48 hours at 4°C, shaking. On the last processing day, the sections were washed 6x5' in 1xPBS, followed by a 2 hour incubation in the secondary antibody diluted 1:200 in immuno buffer. Then the tissue was washed in 6x5' in 1xPBS, followed by 2 hour incubation in an Avidin-Biotin Complex (ABC kit VECTASTAIN Elite Kit, Vector Labs) for HRP conjugated complex, in a 1:1000 dilution in immune buffer (allowed to cross-link for at least 30 minutes before usage). Sections were then washed in 6x5' in 1xPBS and then prepared for the diaminobenzidine (DAB) reaction to produce a dark brown color reaction product visible under light microscopy (5.0 mL Milli-Q H₂O, 2 drops buffer, 2 drops H₂O₂, 4 drops DAB).

Double fluorescent immunohistochemistry: To test for co-expression of Fos/CaMKII α and Fos/pERK, immunohistochemistry was performed. On Day 1, brain sections of the PVN, Lateral septum, Bed Nucleus of the Stria Terminalis and Posterior Hypothalamus were selected and washed 6x5' in 1x Phosphate buffered saline, followed by a 1 hr pre-incubation in block buffer (5% Normal Donkey Serum, 0.5% TritonX-100 dissolved in 1xPBS) . The sections were then washed again 6x5' in 1xPBS and then incubated in the first primary antibody, diluted in immune buffer (2.5% normal donkey serum, 0.5% TritonX-100 dissolved in 1xPBS) for 48 hours, shaking on a platform at 4°C. Rabbit anti Fos (antibody #sc-42; Santa Cruz, CA) was used at a 1:6000 dilution for the most optimal results. On Day 2, the sections were removed from the antibody and washed 6x5' in 1xPBS and incubated in the first secondary antibody for 2 hours

(1:200 concentration) at 4°C, shaking. A donkey anti-rabbit antibody conjugated to AlexaFluor 549 (Jackson ImmunoResearch Labs Inc., WestGrove, PA) was used to detect Fos. Sections were hidden from light with an aluminum foil cover after the first secondary antibody since this antibody is fluorescent and therefore, sensitive to light. Immediately following the first secondary antibody incubation, sections were washed 6x5' in 1x PBS, soaked in 4% PFA for 10 minutes, washed again 6x5' in 1x PBS, and incubated in the second primary antibody for 48 hours at 4°C. CaMKIIalpha (Calcium²⁺/Calmodulin dependent protein kinase – ThermoFisher; #MA1048) was used at a 1:2000 dilution or an antibody against pERK(Cell Signaling; #9101S) was used at 1:4000 dilution for most optimal results. On Day 3, the sections were washed 6x5' in 1x PBS and incubated in the second secondary antibody for 2 hours (1:200 dilution), shaking. To finish, they were washed again 6x5' in 1x PBS, floated under weak lighting conditions in a PBS /gelatin mounting medium (0.5% gelatin and 0.05% chromium potassium sulfate in Milli-Q water) on superfrost plus slides, coverslipped in mounting medium (Vector Laboratories; Vectashield Hard Set Mounting Medium with DAPI #H-1500) and stored in the refrigerator at 4°C until microscopic analysis.

Fluorescent microscopic analysis: A ZEISS fluorescent microscope was used to conduct cell counts with cell imaging by AxioVision, to capture four circular areas around the 3rd ventricle in the posterior hypothalamus at 20 x magnification (see Figure 4 for delineation of the region analyzed). The secondary antibodies used had different fluorescent characteristics (AlexaFluor 488 vs. 549 or 594) that were detected specifically with the combination of dichroic filters on the Zeiss epifluorescent microscope.

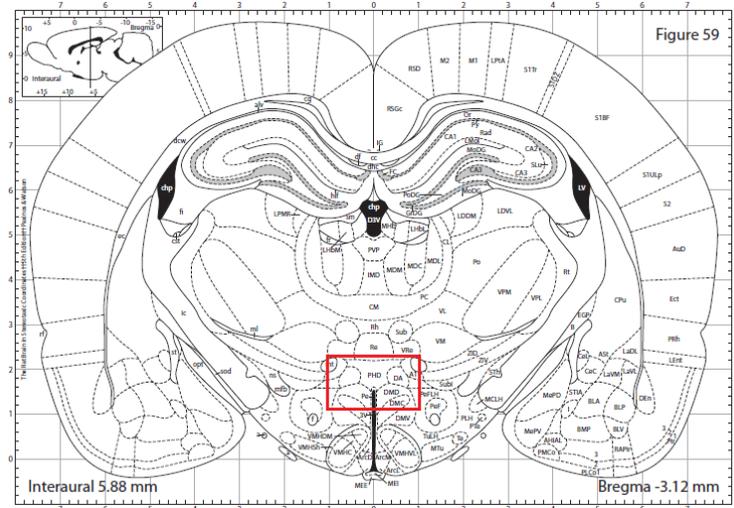


Fig 5: The coronal slice depicting the anatomical region around the anterodorsal posterior hypothalamus. The red box depicts where cell counts were made in 4 different fields surrounding the dorsal 3rd ventricle. This area (Bregma= -3.12) at this level is the posterior hypothalamus.

3.0 Results

3.1 Experiment 1: Finding titres for pELK-1, pERK and CaMKII α while looking at expression in the PH

The titres, the values representing the antibody dilutions at which the antibody is maximally associated with the antigen and can most accurately be detected, were determined by IHC. The optimal titres for the antibodies tested are shown in Table 1.

Fos	1:6000
p-ELK	1:2000
pERK	1:4000
CAM Kinase II alpha	1:2000

Table 1: Shown above are the optimal concentrations of antibodies used for the detection of Fos, p-ELK, pERK and CaM Kinase.

IHC was performed on tissue testing for pElk-1 which has been implicated to activate the transcription factor CREB, but pElk expression could not be observed robustly or consistently. The induction of IEG Fos was minimal in controls and highest at 30 minutes. Strong pERK and CaMKII expression was observed at the level of the posterior hypothalamus. Further experiments focused on these last two protein kinases.

3.2 Experiment 2: Activation of pERK, but not CaMKII is time-dependent

To determine whether the activation of ERK is time-dependent, we measured pERK concentrations in animals that were exposed to 15 minute noise and 30 minute noise as well as a control group (n = 2/group). For pERK cell counts (Table 2), a repeated measures ANOVA was run with the number of sections counted as a within-subjects variable (2) and group as a between-subject variable (control, 15 min, 30 min noise). As expected, no differences were observed between sections within animals, or an interaction with group. However, there was a significant effect of group ($F(1,3)=9.72$ $p<0.05$), as displayed in Figure 5. An LSD multiple means comparison test on the pERK cell counts across groups revealed that the 15 min noise groups was different from both control and 30-min noise groups ($p < 0.05$), which were not different from each other ($p > 0.05$). There was no effect of any factors on CaM Kinase cell counts. Due to problems with tissue availability, the 15 minute time point for CaMKII was not investigated.

Treatment	pERK+/-SEM	CaMKII+/-SEM
Control	34.5+/-1.8	80+/-5.4
15 min noise	69.25+/-11.6	-
30 min noise	39.75+/-3.75	67.75+/-6.5

Table 2. Shown above are protein kinase levels as measured by immunohistochemical DAB reaction. pERK levels rise at 15 minutes and return back to close to baseline levels at 30 minutes of noise exposure. There is no significant difference between control and 30 minute noise group CaMKII levels.

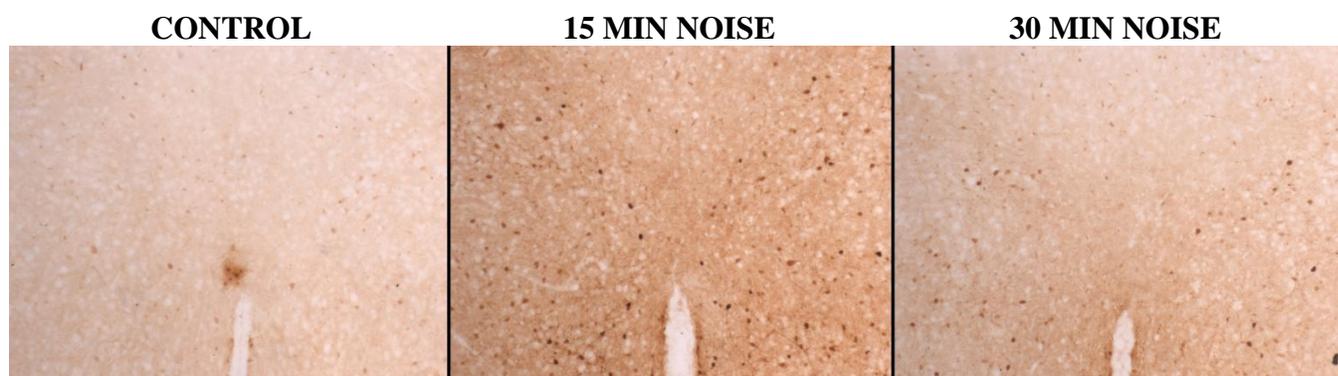


Fig. 5. The pictures depict the area around the 3rd ventricle. pERK levels are at baseline levels in control animals, peak at 15 minutes of noise exposure and regress down at 30 minutes.

3.3 Experiment 3: Co-expression of Fos/pERK was significant but Fos/CaM Kinase was insignificant

An analysis of variance (ANOVA) with group as a between subject factor (control, 30 minute noise) showed a significant induction of Fos protein in the 30 minute noise group ($F(1,3)=38.84, p=0.025$). pERK and Fos displayed a high level of co-expression, as shown in Table 3 and Figure 6 (left panel). However, colocalization of CaMKII into Fos cells was very low, as shown in Table 3 and Figure 6 (right panel).

treatment	Fos+/-SEM	pERK/Fos	Fos +/-SEM	CaMKII/Fos
Control	13.25+/-5.8	11.75+/-5.8	5+/-1.8	.42+/-0.08
30 min noise	143.6+/-45.9	120.67+/-36.3	101+/-7.2	9.58+/-0.92

Table 3. This table shows the means and standard errors of the means for control and 30 minute stress groups Fos expression and co-expression of pERK and CaMKII in Fos positive cells.

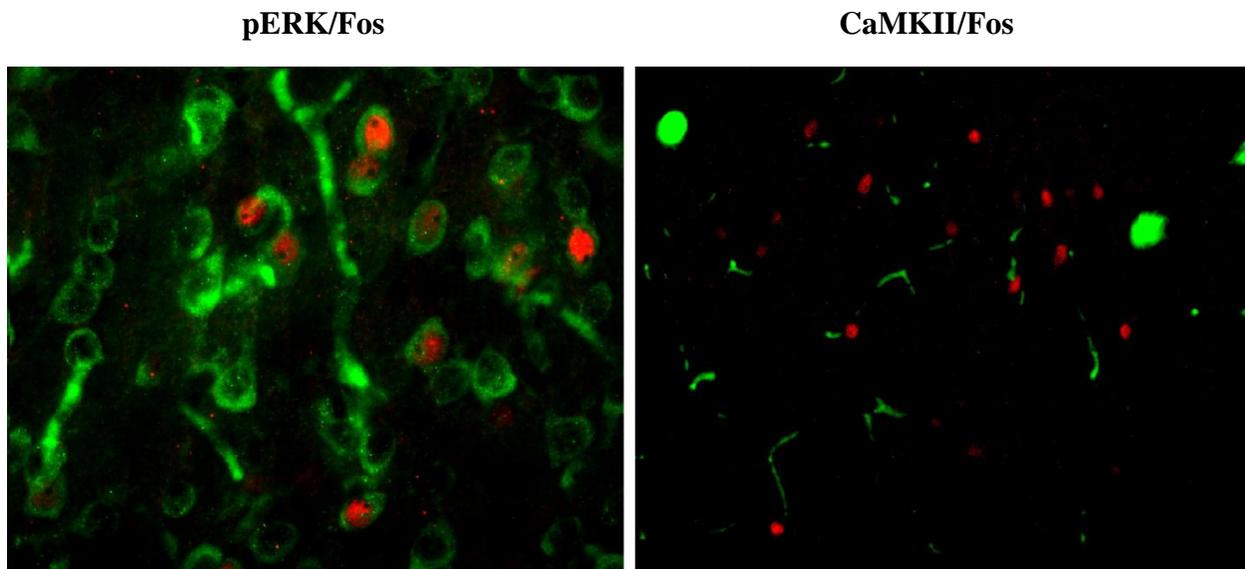


Fig. 6. Above are shown comparative pictures of pERK/Fos co-expression and CaMKII/Fos co-expression in the posterior hypothalamus of stressed rats. Magnification for the left panel is 400X, while magnification in the right panel is 200X. The color red represents Fos while green represents pERK or CaMKII.

4. Discussion

The present study explored the localization of pELK-1, pERK and CaMKII in the posterior hypothalamus of rats following an acute episode of stress.

Elk has been shown to be activated by ERK during plasticity and to induce transcriptional changes downstream via CREB (Tian & Karin, 1999). However, the antibody employed to detect pElk-1 in the current study did not consistently detect a protein product. Since pElk-1 was tested for in tissue perfused traditionally, the inconclusive results may have been affected by a dark or partially visible background. Further investigations can look at pElk-1 expression using the new perfusion method. Because of these technical limitations, no conclusions can be made about the localization /activation of Elk-1 by stress in the posterior hypothalamus.

The discovery of increased phospho-ERK levels in stressed animals suggest that pERK could be part of a signaling pathway that mediates the plasticity associated with the habituation that occurs with repeated exposures to noise. Since noise was the last sensory stimulation the animals experienced before they were sacrificed, and pERK levels were observed to increase in the PH, it can be concluded that the noise exposure directly facilitated this change. The next steps were to measure the expression of pERK at different time intervals of stress and to look for it in cells characterized as active with Fos.

We showed that phospho-ERK levels were highest at 15 minutes of noise exposure and declined at 30 minutes. The time-dependent activation of ERK is congruent with the theory of habituation; ERK signaling takes place shortly after the stimulus (15 minutes) and is turned off with prolonged stress (30 minutes). The PH sends projections to the mpPVN, a site where releasing hormones are secreted, therefore the intracellular plastic changes of pERK can possibly contribute to attenuated HPA axis response in subsequent stress exposures.

The third experiment showed much more Fos expression in stressed animals than controls, but the ratio of co-expression was very similar between the control and stress

conditions. This effect is due to the high expression of pERK in control and stressed animals which indicate that the activation of ERK was caused by factors beyond stress. The novel environment of the acoustic chambers was likely not a source of stress for the control animals because they were allowed one night to adapt before testing day. Similarly, the duration of sacrifice was too short (~1 minute) to have activated signal transduction systems. pERK presence in control animals could mean that ERK is basally phosphorylated, providing some other functions than plasticity. This means that stress-induced phosphorylation of ERK has a very rapid onset and termination and that stress-induced extra phosphorylation is only detectable at a specific time.

A limitation of the study was that due to the extremely high levels of pERK in control and stressed subjects, the exact number of cells expressing pERK could not be manually quantified. A better procedure to determine if there was a difference in pERK levels between control and stressed subjects would be a Western Blot which provides a more accurate quantitative analysis of protein expression.

pERK was widely expressed in active PH neurons, in agreement with the high expression levels we observed with the 15 minute DAB reaction, the pending question now is if we would observe a larger ratio of Fos/pERK co-expression at the 15 minute time point. The next step should be to conduct a between-subjects study to observe the phosphorylation patterns of ERK at additional time intervals from 0 to 45 minutes. Stress exposure should extend to 45 minutes because pERK levels at 30 minutes were still a little bit higher than in baseline controls. Most habituation occurs with the first few stress exposures, therefore phosphorylation of ERK would not be expected in the later stages of repeated exposures.

While the highest pERK levels were shown to be at 15 minutes, Fos levels peaked at ~30 minutes meaning only the Fos mRNA levels would have been detectable at 15 minutes. In a different manipulation of this experiment, to better correlate pERK/Fos co-expression, Fos mRNA and not Fos protein should be measured with in situ hybridization, combined with a subsequent immunohistochemical analysis for pERK. If results are expected and Fos/pERK co-expression is highest around 15 minutes and declines by 45 minutes, it could be directly inferred that the transcriptional changes that lead to altered gene expression reducing the neuroendocrine and autonomic responses to stress may be mediated in part by ERK.

In order to further understand the relationship between pERK and HPA axis habituation, I would conduct a within subjects study observing pERK and Fos expression in the posterior hypothalamus. Rodents would be exposed to 15 minutes of noise for several consecutive days. A subset of the sample size would be sacrificed and used for research each day after the stress exposure. The subjects should be stressed on consecutive days because HPA axis corticosterone habituation only takes place after the second stress exposure, meaning some learning and memory formation has to occur. HPA axis hormones such as ACTH and corticosterone would be measured following every stress exposure. In situ hybridization would be used to measure Fos protein and immunohistochemistry would be used to measure pERK. Fos and pERK levels would be expected to decrease between sessions, as well as HPA axis hormones. This study would help correlate pERK habituation and its possible role in regulating the steady decrease of the HPA axis response following repeated exposures to stress.

In the first experiment, CaM Kinase was shown to be present in the PH in control and stressed animals with DAB IHC. This is expected as this kinase is constitutively expressed in cells and also we were not looking for the activated/phosphorylated state of CaMKII.

Protein levels in the second experiment showed no significant differences between control and stressed animals. A limitation of this experiment was that due to problems with blocking and sectioning, we did not have enough tissue from the 15-minute stress group to use with CaMKII, but the level of expression would not be expected to change because we were not looking for the phosphorylated form of the enzyme.

Double fluorescent immuno studies showed significant but very low or nonexistent co-expression with Fos in control animals vs. stressed animals, which does not support our hypothesis that cells that are active during stress contain the CaMK signal transduction pathway at the level of the PH. Low expression of CaMK may be attributed to the type of secondary antibody used in DAB IHC vs fluorescent IHC. The DAB reaction showed much better expression of CaMKII as opposed to the double fluorescent immuno, suggesting that perhaps the fluorescent antibody was not as sensitive against this protein as the antibody used in the DAB reaction, or the effectiveness of the DAB accumulation via the enzymatic amplification of the peroxidase reaction.

So far the molecular processes explaining plastic changes occurring in response to acute stress remain largely unresolved. Whether this process is LTD or LTP mainly depends on the types of cells, excitatory or inhibitory, that become activated in response to stress, but this level of detail has not been clarified conclusively yet. The limitations of this study provide basis and direction for future investigations and these findings fuel my inquiries to probe further to find a direct relationship between pERK and CaM Kinase in regards to stress habituation. It is difficult to study intermediate signaling molecules due to their pleiotropic nature and only further experimentation can shed light on unexplained intricacies. In order to gauge the role of ERK in habituation and LTP/LTD, it would be interesting to look at whether ERK inhibition would

attenuate stress-elicited habituation. Detection of phospho-CREB would help to further delineate the pathway associated with stress adaptation from ligand binding to nuclear activity.

Measurement of NMDA/AMPA receptor levels before and after repeated stress exposure could provide further evidence whether this process is LTP or LTD related. The measurement of the neurotransmitters signaling from the posterior hypothalamus to the PVN and raphe pallidus by microdialysis could provide insight into the neurotransmitter systems involved and further support either LTP or LTD-like mechanisms at play.

It is important to study the neural mechanisms involved in habituation to stress because the well-known negative feedback mechanisms of the HPA axis do not completely account for habituation. It has been shown in adrenalectomized rats that habituation to a startle response still occurs despite the lack of glucocorticoids that are responsible for the negative feedback loop. (Davis & Zolovick, 1974).

Habituation to different forms of stressful stimuli is an evolutionarily beneficial mechanism. Hans Selye's General Adaptation Syndrome proposes in the Exhaustion Stage that excessive activation of the HPA axis and the autonomic nervous system can be detrimental to an organism. This is supported by numerous conditions like anxiety disorder, post-traumatic stress disorder and depression, which can be caused by an overactive HPA axis. An overactive HPA axis can bring about a feeling of helplessness when stress does come. In the grander scheme of things, repeated exposure to stress is the catalyst for a plethora of psychiatric diseases, therefore understanding the neural mechanism underlying stress adaptation may help understand the etiology of these diseases as well as developing novel medicinal strategies to bridge the bench to bedside gap.

5. References

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