Association of GRK3 with stress resilience and stress susceptibility: Behavioral and pharmacological evidence

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A Dissertation Presentation to

The Department of Pharmacological and Pharmaceutical Sciences

University of Houston

In Partial Fulfillment of

The Requirements for the Degree

Doctor of Philosophy

By

Manish Taneja

June 2011

“For my

parents and

their dreams”

ACKNOWLEDGEMENTS

I would like to thank my mentor Dr Douglas Eikenburg for all his efforts, patience, and guidance in making me a better thinker. He has been instrumental as a mentor and his training has provided me a vision to solve any problem and above that how to ask a right question. Dr Eikenburg is an amazing teacher, his honesty, punctuality and passion towards teaching is undoubtedly beyond comparison. I have thoroughly enjoyed last 4 years of science with positive criticisms. Mentors have the art of converting capabilities of their student into their abilities. I am glad to have some of my capabilities turned into abilities and all credit goes to Dr Eikenburg.

When I came to this department, the first person I met was Dr Kelly Standifer. It has been 5 years that I have known Dr. Standifer and she has been an inspiration to me. She has always created that spark of curiosity, which makes thinking clear, logical and rationale. Her way of teaching has allowed me to vision better and enabled me to contribute to science in a meaningful manner. I thank you Dr Standifer for all of your constructive scientific inputs to my project. I am grateful for what I have learned and will learn from you.

“It is difficult to unlearn the learned things and break the dogma of known science”, I still remember it from Dr Richard Bond’s first lecture. I thank you Dr Bond for making me understand the philosophical rationality of thinking and in some ways to apply it to my project. I would like to thank Dr. Yuen-Sum Lau, for providing all the scientific and technical inputs during my project. In addition, cryostat from his laboratory was the backbone of my project. I thank Dr Lau for allowing me to use his laboratory facilities. I am grateful for his technical expertise in analyzing blinded catecholamine data for my project.

Neurobehavioral assessments related to stress was a significant part of my project. I am grateful to Dr Kevin H Happe for training me in the behavioral

paradigm which was the nuclear core of my project. I would like to thank Dr Mariella De Biasi, for her prompt replies to all my questions regarding these behavioral studies. Her inputs to my project were critical and helpful.

When I joined Dr Eikenburg laboratory, all the techniques were new to me. I would like to thank Samina for providing me all the required hands on experience in the lab. She has been a wonderful colleague in the lab and a very good technical mentor. I would like to thank the entire faculty for their teachings, administrative staff, animal house staff and all PPS graduate students for their help and support in these last 5 years. I would like to acknowledge and thank all other laboratory personnel in the department for allowing me to utilize their laboratory resources as and when required.

I would like to thank Dr DK Majumdar, for all his teachings and above all lighting the candle of big dreams. I have reached this far in my life, thanks to all his encouragements and appreciations.

Very special thanks to my sisters Sheetal, Deepti, Rosy, Manisha, Varsha, Apurva, Mona, and brother Jatin for all their love and support through all these years. I would like to thank my grandparents for believing me and my endeavors. Thanks to my big mom, big dad, my aunt rashmi, and my uncle ajay for all their love and trust. Kanchan and Vimpy, you two have made my world brighter, happier and wonderful. I thank you for your love and bondage. Sumit, Vineet, Nadeem, Amit, and Sanjeev, you are the pillars of friendship. Thanks for always understanding me and accepting me for what I am. Sumit, without you my career in pharmacy would not have been even started, thanks for being the torch bearer. Very special mention to Quaisar (for being patient and caring), Gaurav (for being there always), Kaustuv (for being appreciative), Bajaj (for all those unending talks),and Jyoti (always reminding me of my childhood) guys without your support and friendship, these five years would not have been possible.

And above all, I am thanking all of you because of my parents and their dream to see me succeed in all parts of life. I love you mom; I love you dad.

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ABSTRACT

On exposure to stress, persistent stimulation of alpha$_{2A}$-adrenoceptors (α_{2A}-AR) and corticotrophin-releasing factor 1 receptors (CRF_1-R) increase the receptor signaling in locus coeruleus (LC) and amygdala (two critical brain regions involved in stress response. If this stimulation remains uncontrolled, it initiates the adverse consequences of stress. G protein-coupled receptor kinase-3 (GRK3) mediated desensitization of α_{2A}-AR and CRF_1-R, which terminates the enhanced signaling, is considered an adaptive response. It is not known if there are any biochemical alterations in this adaptive response in stress resilient individuals which make them resistant to the negative consequences of stress.

The purpose of this research was to understand the stress-induced alterations of GRK3 levels and their relationship with α_{2A}-AR/ CRF_1-R levels in LC/ amygdala of stress susceptible and stress-resilient rats. The model utilized, exposure of rats to a single bout of 100 unpredictable inescapable tail shocks, resulted in two distinct behaviors: learned helpless (LH) behavior, (stress susceptible, escape deficits≥ controls) and non-helpless (NH) behavior (stress resilient, escape behavior ≈ controls). A single bout of stress produced readily reversible behavioral changes. Thus, the present study examined time-dependent correlates of GRK3, α_{2A}-AR/CRF_1-R levels from the appearance to the disappearance of LH behavior. In order to evaluate the differences between readily reversible and prolonged LH behavior, a new model of repeated bouts of tail shocks was created. This model showed prolonged behavioral changes.

Finally, the efficacy of desipramine treatment in reversing repeated stress-induced behavioral and biochemical changes was assessed.

LH behavior induced by a single stress was associated with reduced levels of GRK3 and relatively higher levels of α_{2A}-AR /CRF_1-R in LC. These changes appear as early as 1h-post stress and disappear when LH behavior is no longer present. Amygdala showed similar changes compared to LC, but the biochemical distinctions don't appear at 1h post-stress. Instead, the global effects of single stress are seen. Repeated bout of stress (Day 1, 4, 7) generated a bimodal population distribution among stressed rats with prolonged NH and LH behavior (14 days). Levels of GRK3 and α_{2A}-AR/ CRF_1-R were affected similarly to single stress LH rats. In contrast to GRK3, GRK2 also was reduced which was unchanged during single stress. Amygdala showed similar changes after repeated stress compared to LC except that there was no change in GRK2 levels. Desipramine treatment (5mg/Kg bid/14 days) reversed the behavioral deficits of repeated stress LH rats. The levels of GRK3/2 were normalized in LC along with down-regulation of α_{2A}-AR and CRF_1-Rs. Amygdala also showed restoration of GRK3 levels with no receptor down-regulation. Collectively this data suggest that LH behavior (single and repeated stress), an index of stress susceptibility, is associated with reduced GRK3 levels accompanied by relative increase in α_{2A}-AR and CRF_1-R levels in LC. The strength of this association is indicated by the fact that cluster analysis of these proteins, collectively, can predict rat behavior post-stress, independent of behavioral testing.

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ABBREVATIONS

1^0 Ab-Primary Antibody

2^0 Ab-Secondary Antibody

5-HT-5-hydroxy tryptamine

CRF_1-R-Corticotrophin Releasing Factor 1 Receptor

CRF_2-R-Corticotrophin Releasing Factor 2 Receptor

CRF-Corticotrophin Releasing Factor

CUMS-Chronic Unpredictictable Mild Stress

EDTA-Ethylene Diamine Tetra Acetate

EPI-Epinephrine

EPM- Elevated Plus Maze

GPCR- G Protein- Coupled Receptor

GRK- G Protein- Coupled Receptor Kinase

GRK2- G Protein- Coupled Receptor -2

GRK3- G Protein- Coupled Receptor -3

HSP - Heat Shock Protein

LC- Locus Coeruleus

LH- Learned Helpless

MFSS- Modified Forced Swim Stress

Na_2EDTA- Sodium Ethylene Diamine Tetra Acetate

Na_2SO_3 – Sodium Sulfite

NC- Naïve Control

NE-Norepinephrine

NH-Non-Helpless

OFT-Open Field Test

ORL1-Opioid receptor-like 1

PAGE-Poly Acrylamide Gel Electrophoresis

PGi-Nucleus paragigantocellularis

PMSF-Phenyl Methyl Sulfonyl Fluoride

PrH-Nucleus prepositus hypoglossi

PVDF-Polyvinyldifluoride

SDS-Sodium Dodecyl Sulfate

SEM-Standard Error of Mean

SNP-Single Nucleotide Polymorphism

SSRI-Selective Serotonin reuptake inhibitors

TCA-Tricyclic antidepressant

TC-Tested Control

α_1-AR-Alpha$_1$-Adrenoceptor

α_{2A}-AR-Alpha$_{2A}$-Adrenoceptor

β_2-AR-Beta$_2$-Adrenoceptor

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1. INTRODUCTION

According to the World Health Organization (WHO), 25% of the world population at least once in their life time suffers from mental illness. In the USA in 2010 approximately every one in three individuals (≥ 75 million*)* suffers from mental illness with a shocking 57.4% lifetime prevalence rate (more than one in two individuals). The economic burden with direct and indirect involvement is estimated to be $150 billion [1]. Clinical depression is one of the major mental illnesses affecting 15 million Americans every year. It is being predicted that by 2020, depression will be the 2nd most prevalent disease in the world [2]. Stress as a significant factor contributes to depression [3-5].

When Robert Hooke put forth the law of elasticity in the 17th Century, he never would have thought of his law being applied to the human biological machine. He stated that when “load” is applied to a system , it experiences “stress” which results in development of “strain” on the system at two levels; a) elastic deformation wherein energy generated by load (opposing forces) is completely absorbed by the system such that when load is removed the original non-stressed state of the system is acquired again, and b) plastic deformation where physical strain permanently damages the system and the system does not regain its original state , even after the load is removed. The ratio of elastic to plastic deformation is the property of the material of the system which is

experiencing stress due to load exposure [6]. Cox (1978) took this law of elasticity and perfectly applied this analogy to biological systems, suggesting that every individual has a degree of resistance to stress (like the elastic limit to deformations in physical systems on experiencing stress) [7]. This inbuilt resistance can prevent against the damaging effects of a certain level of stress, but when it crosses the threshold of the individual, permanent damage occurs (physiological and psychological).

The degree of resistance to stress is an integral part of an individual, and varies from person to person. This is why individuals show differential capability to cope/adapt with stress and form a distribution in a population ranging from high degree (resilient) to low degree of resistance (susceptible) towards the negative consequences of stress. The human machine is different from mechanical machines as it is exclusively the perception of an individual which makes an event stressful or non-stressful. The perception of an event is ultimately determined by an individual's susceptibility and resilience patterns which form the basis of either developing or not developing stress related disorder. ***The big question is, are there biochemical, physiological, and or psychological differences in resilient individuals which make them resistant or able to adapt to stressful conditions without developing any negative consequences of stress?***

Stress is a condition when there is an imbalance between actual or perceived demand. In order to correct these imbalances, the body makes biochemical alterations which are then manifested as modified behavioral responses. It is the brain which coordinates the stress response by deploying multiple stress mediators viz; neurotransmitters (e.g. noradrenaline (NE) serotonin, corticotrophin releasing factor (CRF), dynorphins) and hormones (e.g. cortisol /humans and corticosterone/rodents, angiotensin). Utilization of these many stress mediators in various temporal compartments in brain viz; cortex, amygdala, hippocampus, hypothalamus, dorsal raphe nucleus, and locus coeruleus (LC) adds another level of complexity to an orchestrated stress response [8-10].The brain regions critical to stress response are: LC, a primary recipient of stressful stimuli and amygdala, which emotionally tags the stress response. LC and amygdala also are important because these regions have functional reciprocal inter-connections mediated by two major neurotransmitters, CRF and NE [11, 12]. CRF and NE mediate their action via G protein-coupled receptors (GPCRs), the corticotrophin releasing factor-1 (CRF_1- R, excitatory) receptors and alpha$_{2A}$-adrenoceptors (α_{2A}-AR, inhibitory), respectively, and both GPCRs are present in LC and amygdala.

Stress exposure increases NE and CRF in LC and amygdala, which persistently stimulates α_{2A}-AR and CRF_1-R respectively. Termination of persistently stimulated GPCR signaling is an adaptive response to stress,

contributing to the protection of neuronal cells from the pathophysiological consequences of stress-related disorders. The most important step in terminating the signaling of a stimulated receptor is desensitization of the agonist-activated GPCR by GPCR kinase (GRK) mediated receptor phosphorylation. α_{2A}-AR and CRF_1- R are preferentially desensitized by GRK3 (1 of 7 identified isoforms of GRKs) [13-15].

Several pieces of evidence suggest that GRK3 is an important modulator of the stress response which might govern the susceptibility of individuals to stress. 1) Outside of the olfactory system; LC is the place where GRK3 is expressed at higher levels as compared to other brain regions involved in stress response [16]. The GRK3 regulated α_{2A}-AR and CRF_1- R are abundant in LC; 2) A single nucleotide polymorphism (SNP -382 G/A) in the promoter region of the human GRK3 gene is present in a sub-set of patients suffering from bipolar disorder [17-19]; 3) Lymphocytes of the subset of patients expressing this polymorphism exhibit reduced levels of GRK3. Individuals with this polymorphism exhibit a higher propensity towards stress initiated relapses of BPD [19] ; 4) Lower levels of GRK3 were observed in frontal cortex of BPD patients [20] ; 5) GRK3/2 levels were significantly reduced in platelets of major depressive disorder patients [21]; and 6) Chronic administration of lithium (mood stabilizer) to rats selectively increases levels of membrane associated GRK3 protein in frontal cortex [22]. Taken together this evidence suggests that GRK3-promoted

desensitization is an adaptive mechanism involved in controlling α_{2A}-AR and CRF_1- R signaling and a decline in GRK3 results in loss of adaptation that can potentially increase an individual's susceptibility to develop stress-related disorders.

Similar to the differential response to stress in humans, rats subjected to inescapable stress (unpredictable tail shocks while restrained) also exhibit stress-resilient and stress-susceptible behaviors. One group exhibits prolonged escape latency (24h post-stress) as compared to controls in an escapable mild shock condition , a phenomenon called learned helplessness (LH-stress susceptible) [23-25]. The other group exhibits escape latency similar to control and these rats are called non-helpless (NH- stress resilient). Thus, this model has the potential to answer a variety of questions regarding differences in regulation of α_{2A}-AR and CRF_1- R by GRK3 in stress-resilient and stress-susceptible populations in two prime brain regions, LC and amygdala.

A single bout of stress in rats induces LH behavior for 2-3 days only, after which the behavior reverses to that observed in normal unstressed animals. The effects of single stress do not translate into prolonged behavioral changes. It is not known whether repeated inescapable stress induces prolonged LH behavior and, if so, if it relates to regulation of GRK3. This raises an interesting question of differences between single vs. repeated effects of stress. Namely, in repeated stress are the observed changes simply the additive effect of single stress

episodes or are the changes after repeated stress completely different from single stress?

STATEMENT OF PROBLEM

It has been postulated that loss of adaptation to stress may lead to a higher propensity towards stress susceptibility. The mechanism underlying this loss of adaptation changes is still not known. The goal of the present research is to examine the relationship between stress and adaptation and to begin to develop an understanding of the cellular mechanisms involved in determining resilience and susceptibility to stress. The hypothesis to be tested is that stress-induced modifications in α_{2A}-AR and CRF_1- R signaling in locus coeruleus and amygdala of stress susceptible (learned helpless) individuals are associated with changes in GRK3 levels was tested. As a part of study the temporal and spatial correlates of α_{2A}-AR/CRF_1- R and GRK3 from the appearance to the disappearance of readily reversible LH behavior induced by a single bout of stress of single stress were examined. A second hypothesis evaluated was whether the changes at the level of α_{2A}-AR/CRF_1- R and GRK3 are different when a single bout vs. repeated bouts of stress causes LH behavior. A validated model for prolonged LH behavior was generated by subjecting rats to repeated bouts of stress and then examined the differences at the level of α_{2A}-AR/CRF_1- R

and GRK3 between single and repeated stress. A third hypothesis evaluated whether a pharmacological intervention can reverse behavioral and biochemical changes induced by repeated bouts of stress. Desipramine treatment was used to study the reversal of prolonged LH behavior induced by repeated bouts of stress and relation of changes in α_{2A}-AR/CRF_1- R and GRK3 levels with prolonged LH and NH behavior was further evaluated.

The significance of this study is that ***GRK3 may appears to act as an "adaptation switch"*** for balancing the CRF_1 (excitatory) and α_{2A} (inhibitory) receptor signaling in response to stress. Restoring GRK3 function might not only help in providing resilience to a stress susceptible individual, but may suggest new avenues in drug discovery that target GRK3 or mechanisms that regulate GRK3 levels as a way to restore control of the responses of α_{2A}-AR and CRF_1-R and thereby prevent or reverse stress-related pathologies.

2. LITERATURE REVIEW

2.1 Stress resilience and stress susceptibility

Stress is defined as a parallel yet sequential set of compensatory orchestrated events arising from incongruity between expectations (realized from a current situation, coded by an individual's genetic makeup or extracted from past learning experiences) and perceptions (inferred from the external or internal milieu) in a given set of circumstances [26]. Stress is a predisposing factor for approximately 75% of all illnesses [27]. However, it does not affect all individuals similarly. It is well identified that individuals have differential ability to cope with stress, which is a determining factor in characterizing resilience or susceptibility to the negative consequences of stress [28-30]. All major stress-related pathologies affect brain neurochemistry, which then becomes the epicenter of maladaptations leading to disease.

A major understanding of stress and its implication in disease, particularly neuropsychiatric disease, comes from animal models. In rodents, tail shock stress (TSS) [31], chronic unpredictictable mild stress (CUMS) [32], and social defeat paradigms [33] are models where application of a stressor results in behavioral changes that identify two populations from the same set of stressed rodents. One population behaves similarly as controls (stress-resilient) while the other behaves significantly different from controls (stress-susceptible). Most of

the neurobiological research in animal models has focused on the stress susceptible populations and how pharmacological interventions prevent or reverse the stress susceptible phenotype. In contrast, there has been very little attention given to identification of the neurobiological mechanisms mediating stress resilience.

Our body is always threatened by external or internal stimuli (stressors) which can potentially disrupt the body's homeostasis (maintenance of oxygen tension, temperature, and pH and glucose supply) vital for functioning of the body. Biological systems that maintain homeostasis by changing their functional capacity are termed allostatic systems (maintaining homeostasis by change), and for our survival these are considered adaptive [34, 35]. Three conditions can arise from an encounter with stress: First, if the allostatic system that was activated was properly controlled by the normal functioning of the body, the condition is called eustais. Second, if the allostatic system brings the homeostatic system back to normal at the expense of controlled utilization of available resources, the condition is called allostasis. Third, the allostatic system can cause the homeostasis to reach a newer improved status from the experience gained from the stressful experience; a condition called hyperstasis (improved homeostasis) [4]. Allostasis, within limits, is beneficial for the survival of a species .However, if it goes out of control it becomes a burden. When allostasis is sustained, it is called an allostatic state. The body can sustain this allostatic

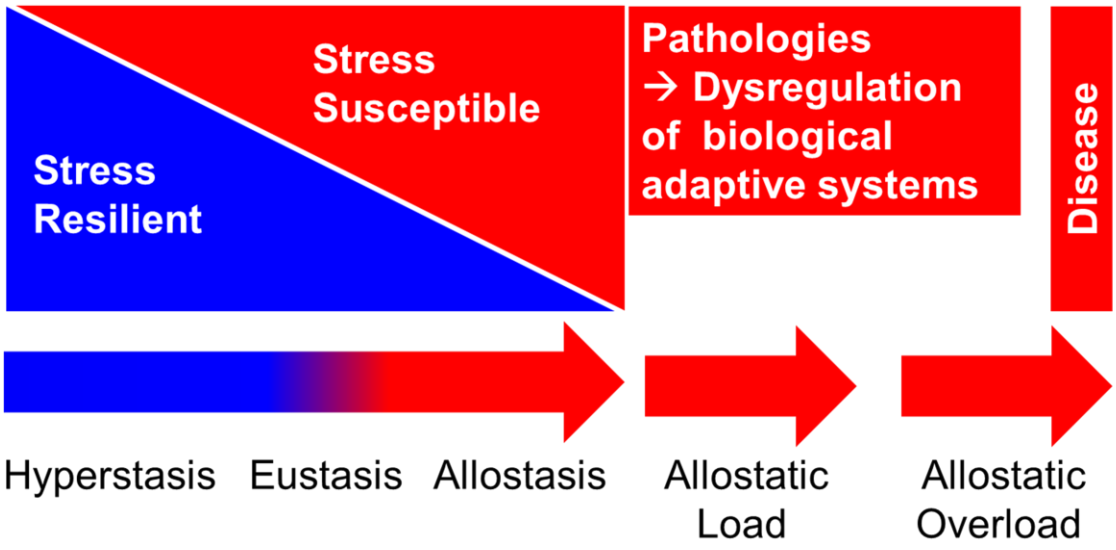


Figure 1: <u>Stress susceptibility and disease</u>

state for only a limited period of time, during which it fuels the maintenance of homeostatic systems by another source of energy. When maintenance of this homeostatic state leads to wear and tear of the body, the condition is called allostatic load (cost paid by the body for maintaining allostasis). The damaging effect of wear and tear leads to an altered homeostatic state. This additional load on the biological system forms the basis for the development of stress-related pathophysiology (for e.g. a person under job stress is diagnosed with high blood pressure (allostatic load) , continues to eat a high fat diet (allostatic overload) resulting in the development of atherosclerosis and increased risk for stroke or myocardial infarction) [34-36]. Mechanisms involving transformation

from hyperstasis to allostasis give rise to a continuum from resilient to susceptible phenotypes depending on the extent of deployment of adaptive stress responses at multiple phenotypic levels **(Fig 1)**. When allostasis remains ungoverned in stress susceptible populations for longer periods of time (acute to chronic stress condition) a system overload condition is created making the body work beyond its capacity. The uncoordinated overload causes system breakdown and pathological symptoms turn into a full blown disease.

The question is, is stress resilience just non-occurrence of maladaptive changes or is there a third side to our understanding of the stress coin? Developmental studies suggest that epigenetic influences on an individual's genes are dynamic events which modify neural circuits / functions resulting in increasing the range of neuronal structural/functional capacity. The extent of the increased strength of these integrated circuits determines the extent of stress resilience. Therefore it can be considered that stress resilience is not only absence of stress-related pathologies, but also a dynamic process that is active throughout human life.

2.2 Acute and chronic effects of stress

Stress is a response of an individual based upon their perception towards a potential threatening condition. This stress response requires instant behavioral modifications which are accomplished by tuning neuronal functioning at different

levels of the central nervous system. This includes levels which govern cognition (prefrontal cortex), learning /memory (hippocampus), arousal (locus coeruleus) and emotional (amygdala) responses. The duration and frequency of a stressor particularly governs the nature of neuronal responses in orchestrated temporal and spatial compartments [9].

The body generates differential responses depending on the exposed duration (acute or chronic) and frequency (single or repeated) of stress. Acute effects of stress involve rapidly activating neurons leading to fast neuronal transmission and increased hormone release. This is followed by rapid return to baseline levels. It has been seen that on a majority of occasions once the stress is removed, the response to stress is inactivated. Although the effects of the stress are terminated, temporary activation of neuronal populations like those in hippocampus and hypothalamus results in genetic modifications, which in turn changes neuronal responses and may prepare the brain well for further encounters with similar kinds of stressful experiences. In contrast, chronic effects of stress induce prolonged and advancing modifications of gene expression, neuronal structure, and neuronal firing all over the brain. If these changes are sustained for longer periods of time, it causes deviances from normal neuronal network function [5, 9, 34, 37-39]. The influence of duration and frequency of the stressor on the magnitude and pattern of the stress response becomes more and more complex when the varieties of influential factors are taken into account. For

example, type of stress (physical/ psychological), context of stress (time /surroundings), age at which stress is received (newborn/ adult /old),

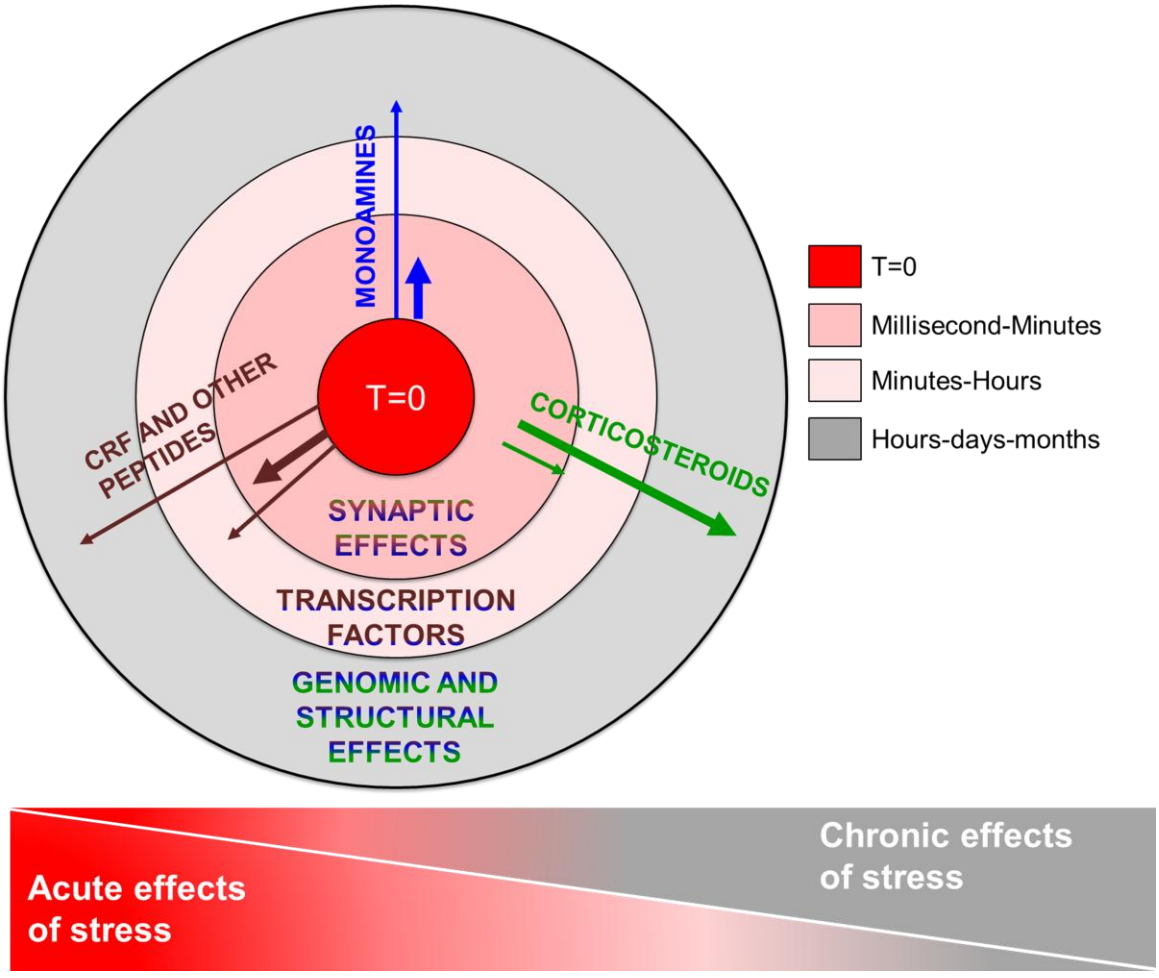


Figure 2: <u>Time dependent profile of co-ordinated stress response</u>
Series of vectors denoting stress onset and each mediator has its own unique vector with specific direction and magnitude associated with synaptic, transcriptional, genomic and structural event. The figure is modified from Joels, M. and T.Z. Baram**.**The neuro-symphony of stress**,** 2009**,** Nature reviews, Neuroscience, 10(6), 459-66.

sex of individual, and genetic constitution with epigenetic influence, all contributes to a greater level of complexities. The array of a stress response utilizes several mediators in a timely fashion, producing early to prolonged effects, with every mediator acting in a unique neural compartment **(Fig 2)**.

2.3 Stress and brain

In brain, stress activates locus coeruleus (LC) -noradrenergic neurons. The activation of LC results in increased norepinephrine (NE) release in efferent neuronal sites. The LC has diffuse projections to many nuclei of the forebrain. The major targets pertinent to stress are hippocampus, prefrontal cortex, dorsal raphe nuclei (DRN) and amygdala which further project their neurons to hypothalamus [8, 12, 40].

Although the primary neurotransmitter of the LC is NE and LC function is regulated by NE, the LC also is affected by various neurotransmitters/ hormones that participate in the stress response. Evidence suggests a complex interaction of corticotrophin-releasing factor (CRF), endogenous opioids, glutamate, and gamma-amino butyric acid (GABA) mediated influences on LC. The result of these interactions is to adjust the activity and reactivity of the LC-NE system in order to mediate adaptive responses (biochemical/neuronal/ behavioral) to stress [8, 12, 40].

The mechanisms of regulation of LC function by these various neurotransmitters/ hormones are almost as numerous as the substances themselves. Glutamate and gamma-amino butyric acid (GABA) mediated influences on LC by their ion channel receptors either excite or inhibit LC neurons, respectively. Cortisol affects LC neurons primarily by altering the expression of genes within the LC; more likely contributes to the longer-term effects of stress. Others substances like endogenous opioids, NE and corticotropin releasing factor (CRF) modulate LC function via the activation of G protein-coupled receptors (GPCR) [12, 41, 42]. As the modulation of GPCR function by GRKs is the primary focus of this study, the remainder of this discussion will focus on the role of GPCRs in regulating LC.

Endogenous opiates can influence the LC via a wide variety of GPCR in LC including the inhibitory mu- and kappa-opioid receptors and an orphan opioid receptor named the nociception receptor (also known as the opioid receptor-like 1, ORL_1). It is postulated that the mu- and kappa-opioid receptors participate in returning the enhanced LC neuronal activity observed during stress to the normal pre-stress condition by reducing the extent of tonic firing in LC [40, 43-45]. The inhibition offered by endogenous opioids protects the neurons from negative consequences of stress-induced activation of LC. This inhibitory effect can be completely blocked by intra LC administration of opioid antagonist (naloxone), suggesting that the effects are modulated by mu-opioid receptor [46, 47]. The

kappa opioid receptor (KOR) is present on presynaptic axon terminals in the LC and thereby inhibits the majority of input (afferent) signals to LC. KOR presynaptic inhibition not only reduces the ability of stimuli to activate phasic tone in LC, but also reduces the tonic activation of LC neurons. Phasic activity is required for focused attention in a situation while tonic activity promotes general scanning behavior. Thus presynaptic inhibition of LC by KOR activation modifies the nature of environment monitoring by modifying LC activity [48-53]. Besides the Mu- and KOR, an orphan opioid receptor ORL_1 also is implicated in inhibiting LC neuronal firing. Nociceptin (endogenous agonist for ORL_1 receptor) deficient mice shows stress susceptible phenotypes with impaired adaptive responses to repeated exposure of stress. Studies in nociceptin and ORL_1 knockout mice emphasize their role in stress susceptibility and drug abuse [54-58].

While the participation of the opiate receptors in the regulation of LC functions in stress in indisputable, the two GPCRs in LC that are known targets for antidepressant drug therapy are the CRF_1 receptor and the $alpha_{2A}$-adrenergic receptor (α_{2A}-AR). Increased neuronal firing from LC activates $alpha_1$-adrenergic receptors (α_1-AR) on CRF containing neurons of amygdala and hypothalamus which project back to LC and thus create a feed forward loop of heightened neuronal firing. This allostatic condition, uncontrolled, results in pathophysiological sequelae. Amygdala not only gets inputs from LC but also

feeds projections to LC, thus making a functional neuro-anatomical circuit with reciprocal regulatory interactions [8, 12, 40] **(Fig 3)**.

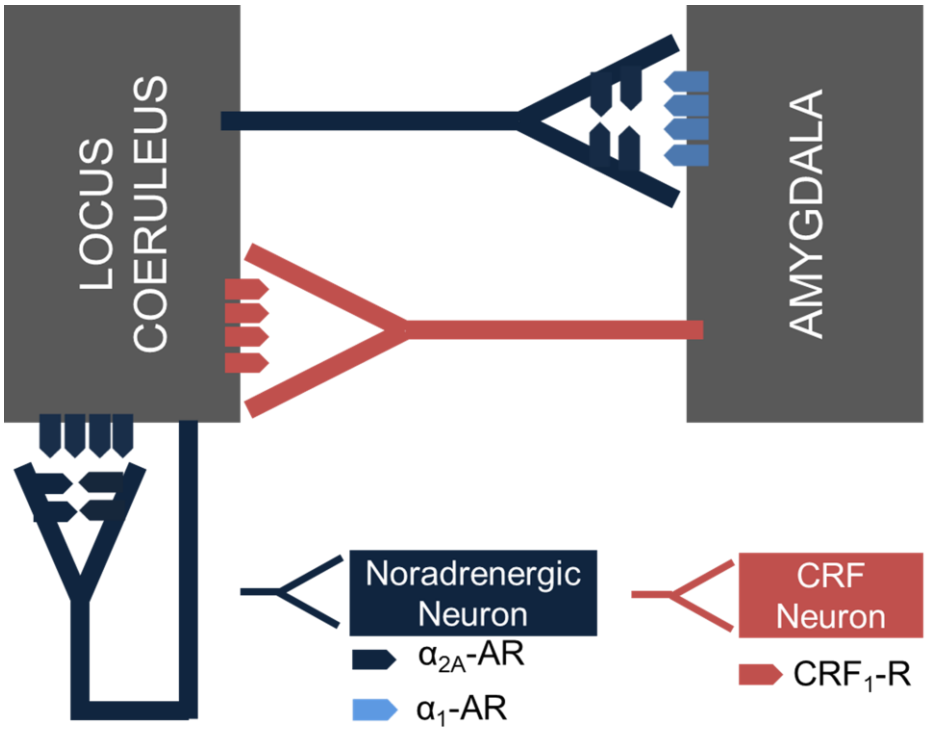


Figure 3: <u>Feed forward loop between locus coeruleus and amygdala</u>

The LC projections to amygdala are essential for memory of aversive events and norepinephrine released from LC efferents in basolateral amygdala help to consolidate aversive learning [39, 59-61]. When the perceived stress is

cued with memory of aversion, it is beneficial in order to provide escape strategies for further encounters under similar stress conditions. Thus, the similarity between LC and amygdala (facilitatory effect over the stress response, both having excitatory influence on hypothalamus) and feedback reciprocal projections makes this an important brain circuit that is involved in mediating the stress response. The involvement of CRF in LC-NE system regulation of the amygdala is critical for the interaction of the functional neuro-anatomical circuit between LC and amygdala. During stress CRF release from amygdala is increased and CRF neurons from this brain region innervate LC. CRF activates CRF_1 receptor (CRF_1-R) on LC and its dendritic fields which further stimulates LC neuronal firing **(Fig 3)**.

The LC also has an abundance of α_{2A}-AR, present in both presynaptic and postsynaptic locations. NE activation of postsynaptic α_{2A}-AR on LC neurons inhibits neuronal firing while activation of presynaptic α_{2A}-AR on recurrent collateral neurons inhibits the release of NE onto LC neurons, which will reduce the activation of inhibitory postsynaptic α_{2A}-AR [62, 63]. The α_{2A}-AR provides inhibitory control on LC neurons for modulating the output of neuronal signals. Therefore, the net output of LC noradrenergic input to other brain regions is determined by the level of activation of varied inhibitory and excitatory receptors present in the LC.

2.4 GRK3, α_2-AR and CRF_1-R: Combined existence in locus coeruleus

The LC is like an integrated chip (IC) in an electrical circuit, that has many regulatory inputs and the unique configuration of the IC (LC's unique biochemical and neuronal configuration) modifies the output in response to a stimuli. Thus LC can be seen as the region in brain, which not only initiates divergent neuronal signals to different brain regions, but also modifies the output of converging signals coming from different regions, in order to refine the tuning of neuronal firing in response to stress (**Fig 4)**.

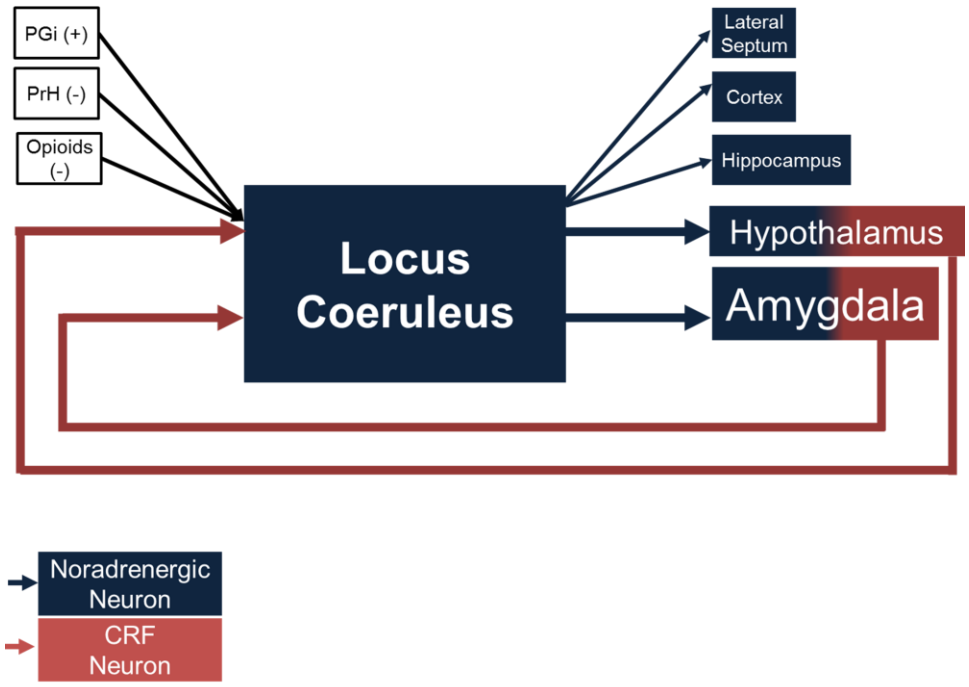


Figure 4: <u>Locus coeruleus, the point of convergence and divergence during stress</u> (PGi- nucleus paragigantocellularis-Glutametergic; PrH, nucleus prepositus hypoglossi-GABAergic)

Stress exposure changes the noradrenergic neuronal firing pattern of LC from one of a moderately tonic (modulated by glutamatergic influence) to a highly tonic pattern. This prevents phasic firing (behavioral readout – from focused attention to general scanning behavior of the environment). This shift is regulated by CRF neuronal projections from amygdala activating postsynaptic CRF1-R in LC [12, 40]. The LC's noradrenergic projections to amygdala and hippocampus cause release of NE and increase the activity of neurons in these regions in order to extract and consolidate information (emotional memory tagged by amygdala and memorized by hippocampus) related to stress [64].

CRF and NE which determine major behavioral responses to stress act via α_{2A}-AR (inhibitory) and CRF_1-R (excitatory) are regulated by GRK3. These two receptors are known targets for antidepressants and modulation of these receptors has the potential to govern stress susceptibility [13-15, 65-68]. Interestingly, the KOR and ORL_1 receptor are also regulated by GRK3 along with mu-opioid receptor which is regulated by GRK2 but cross talk with ORL_1 receptor involves GRK3 mediated regulatory component [48, 69-71]. Modulation of these opioid receptors is implicated in stress and vulnerability towards drug abuse. GRK3 can thus be central to not only in determining the susceptibility and resilience to stress related pathologies, but also to drug abuse. In addition, the predominant GRK in brain is GRK2 and both GRK2 and GRK3 belong to the same family of GRKs. However, outside the olfactory, GRK3 (1 of 7 identified

isoform of GRKs) is only highly expressed in LC [16]. Thus it is not a coincidence that receptors viz; kappa-opioid /ORL_1, α_{2A}-AR, and CRF_1-R, which are acted on by neurotransmitters in the LC are regulated in coordinated manner by virtue of a common underlying factor, namely GRK3.

2.5 Changes in GRK3 levels modulates α_{2A}-AR and CRF_1-R signaling

During stress, increase in CRF and NE levels persistently stimulates α_{2A}-AR and CRF_1-R. GRK3 mediated desensitization (*phosphorylation of an agonist-activated GPCR which increases receptor affinity for arrestin, and inhibits G-protein/receptor interaction*) of α_{2A}-AR and CRF_1-R terminates the enhanced signaling. This is considered an adaptive response to overstimulation of receptors [13-15, 65-67]. Thus, changes in the level of GRK3 can potentially affect α_{2A}-AR and CRF_1-R signaling and consequently stress susceptibility/ resilience as both these receptors are involved in mediating the stress response.

An increase in GRK3 levels by 2 fold in human neuroblastoma cells (BE-2C) makes α_2-AR 70-fold more sensitive to down-regulation. However, inhibiting the function of GRK3 prevents agonist-induced down-regulation of α_2-AR [14]. A 50% reduction in GRK3 levels in Y-79 cells (retinoblastoma cell line) decreases desensitization of CRF_1-R by 65% [13, 67]. Therefore a small decline in GRK3 will increase α_{2A}-AR and CRF_1-R signaling. As neuronal firing is stimulated by CRF_1-R and inhibited by α_{2A}-AR, the consequences of reduced GRK3 may tip

the balance between stimulatory and inhibitory tone of neuronal firing, contributing to the pathophysiological consequences of stress. It can be argued that GRK3 as an “adaptation switch” can balance the activity of neuronal outputs by modulating α_{2A}-AR and CRF_1-R activation/ deactivation kinetics during stress.

2.6 Regulation of neuronal GRK3 levels

GRK3 levels in neuronal cells are regulated by several mechanisms, which influence its transcription, stability, and degradation. In neuronal cell lines CRF induced chronic activation of CRF_1 receptors up-regulates GRK3 expression [67, 72] . Similar to CRF, another stress hormone epinephrine (EPI) also up regulates GRK3 expression by chronically activating α_2-AR and $beta_2$-adrenoceptors (β_2-AR) simultaneously. Both processes increase GRK3 levels by increasing transcription via ERK1/2 activation [72, 73]. Within 15 minutes of receptor activation an increase in GRK3 message (mRNA) can be detected while it takes 6-8h for increased GRK3 levels to be detected [72, 73]. It can be posited that GRK3 up-regulation by chronic activation of the same receptors (α_2-AR and CRF_1-R) which GRK3 down-regulates might be a cellular negative feedback process for maximizing α_{2A}-AR or CRF_1-R desensitization during prolonged exposure to high CRF, NE, or EPI during stress.

Down-regulation of GRK3 involves mechanism related to decreases in stability and increases in degradation. Heat Shock Protein 90 (HSP 90) is a

molecular chaperone that influences stability of the GRK3 protein. Evidence from our laboratory suggests that there is a direct interaction between endogenous GRK3 and Hsp90 protein in human neuroblastoma BE-2C cells. When this interaction is disrupted, GRK3 is targeted for degradation via the proteasomal pathway. Our laboratory also has reported that neuronal GRK3 levels are reduced by calcium-dependent proteolytic degradation via activation of calcium-activated proteases, calpains[74]. Therefore, mechanisms increasing GRK3 might prove beneficial during overstimulation of α_{2A}-AR and CRF_1-R, while decline in GRK3 levels can cause exaggerated α_{2A}-AR and CRF_1-R receptor signaling which can contribute to the development of stress related pathophysiology.

2.7 GRK3: Contradictions in its role during stress

Besides the classical mode of desensitization of GPCRs by GRKs, there are alternate signaling pathways which utilize arrestin recruitment as scaffolding mechanisms for MAPK activation to enable rather than terminate signaling. This ability to enable signaling has been shown to be associated with the negative consequences of stress. During repeated forced swim stress (FSS), GRK3 knockout mice show reduced immobility compared to their wild type counterparts. Immobility in FSS is indicative of behavioral despair and reduction in this behavior is considered a sign of stress resilience, suggesting that loss of

GRK3 prevents the negative behavioral consequences of stress [75, 76]. However this is in contradiction to the classical role of GRK3 wherein it is required to prevent overstimulation of receptors during stress.

Additional studies in GRK3 knockout mice may provide the explanation for this contradiction. Besides CRF, NE, and EPI, the endogenous opioid agonist dynorphin, also is increased during stress and dynorphin activates the KOR [77], which is preferentially regulated by GRK3. Administration of KOR antagonists in nucleus accumbens (NAc) decreases stress susceptibility, while activation of KOR by agonists such as dynorphin results in dysphoria (aversive event) and increases stress susceptibility [75, 77]. GRK3 phosphorylation of Ser_{369} in the C-terminal domain of KOR results in the association of arrestin to the phosphorylated KOR. This results in phosphorylation and simultaneous activation of p38 MAP kinase. Activated p38 MAP kinase is required in NAc for producing the dysphoric effects of KOR agonists. Dysphoria induced by KOR agonists is abolished by over-expression of dominant-negative GRK3 in NAc, which further supports GRK3 being required for facilitating p38 activation in NAc and for the negative consequences of stress [75, 76, 78].

KOR activation in spinal cord produces analgesia; desensitization of KOR in a spinal cord trauma model results in neuropathic pain. Since desensitization of KOR in spinal cord is eliminated in GRK3 knockout mice, no neuropathic pain is observed if spinal cord trauma is induced in these animals [79, 80]. These

reports thus suggest that GRK3 serves different functions in different CNS regions. Signaling by the same GPCR is abolished in some regions while being enabled in other regions. Thus studies in GRK3 knockout mice have the limitation that GRK3 is knocked out everywhere and prevention of aversive effects by NAc activation during stress by loss of GRK3 might lead to erroneous conclusions about GRK3 function in other brain regions during stress. Understanding the role of GRK3 in specific brain regions, therefore, requires studying of regional changes of GRK3 during stress as well as methods that enable regionally targeted manipulations of GRK3 levels.

2.8 GRK3 and mood disorders

Evidence from the clinical literature supports a link between GRK3 and stress, major depression and bipolar disorder. There is a subset of bipolar disorder (BPD) patients who exhibit a single nucleotide polymorphism (G-382N) in the promoter region of the GRK3 gene. Lymphocytes derived from these patients revealed that selective GRK3 expression was reduced compared to controls. The magnitude of reduced expression was inversely related to the severity of patient symptoms [17-19, 81]. It can be postulated that reduced GRK3 levels in the brains of these patients decreased their adaptability to stress and made them more susceptible to stressful stimuli, thus initiating pathophysiological

sequela. This is further supported by the report that these bipolar patients are more susceptible to stress-initiated relapses [82].

The importance of GRK3 in stress related psychiatric illnesses is further highlighted by the following observations; 1) Important stress mediators such as stress NE, EPI and CRF act via receptors (α_{2A}-AR and CRF_1-R) that are preferentially regulated by GRK3 [13, 14, 67]; 2) GRK3 levels are significantly reduced in frontal cortex of BPD patients [20]; 3) GRK3 was modestly, but significantly, reduced in platelets of major depressive disorder patients [21]; 4) Cortical prejunctional α_{2A}-ARs were increased in patients with major depression ; these results could be associated with reduced GRK3 expression [83, 84]; 5) Chronic administration of the mood stabilizer lithium selectively increased membrane GRK3 protein in frontal cortex of rats. If this also occurs in patients with BPD that respond to lithium, it may explain some of lithium's therapeutic actions [22]. Taken together, this evidence supports a protective role of GRK3 in mood disorders.

2.9 Animal Model for stress susceptibility and resilience

Inescapable unpredictable stress in rats generates a differential behavioral response which is apparent when the same stressed rats are subjected to a subsequent milder escapable stressor. The rats showing significantly higher escape deficits compared to non-stressed rats in an escapable situation are

termed learned helpless (LH-stress susceptible). The rats that respond similarly to non-stressed rats are termed non helpless (NH-stress resilient) [23-25, 29]. The LH paradigm is a behavioral model which is extensively utilized for screening antidepressant drugs. The model provides very good predictive validity, as clinically effective antidepressant drugs prevent or reverse LH behavior. However, to date a majority of the research in this model has focused attention on prevention and/or reversal of LH behavior rather than looking at the mechanisms responsible for NH behavior.

There are two major variations of the model for stress-induced LH behavior reported in the literature. In the first variation, the inescapable unpredictable stress and the subsequent mildly stressful escape testing are done in the same context, (shuttle box - foot shocks for both the unpredictable inescapable stress and for the escape testing) [31, 85-87]. In the second variation, the animal is placed in a restrainer (inescapable) and shocks are given (unpredictable) by attaching electrodes to the tail; escape testing is done in a shuttle box (different context) [85, 88]. Both variations of the model are used to test antidepressant efficacy but there are differences in these two variations which must be considered when we are answering questions related to stress susceptibility and resilience. In the tail shock stress (TSS) LH paradigm (no common cues shared between the stressor and escape testing task) the escape deficit is short lived and independent of contextual fear [85, 88]. However, when

exposure to stress and escape testing occur in the same context, escape deficit is long lasting and confounded with contextual fear [85-88] . Another important difference between the variations of the model is the effect that exercise training of rats has on the incidence of rats becoming stress susceptible after being exposed to stress [85, 87, 88]. Exercise training does not reduce escape deficits when the inescapable, unpredictable stress and the subsequent escape testing are done with in the same context. However exercise training reduces escape deficits when stress and subsequent escape testing are done in different contexts (TSS-stress and Shuttle box –escape testing).

2.10 Locus coeruleus and learned helplessness

The simultaneous occurrence of LH behavior and marked activation of the LC noradrenergic neurons after application of inescapable shocks supports the hypothesis that the LC contributes to helplessness. Increased neuronal activity in LC subsequently increases activation of noradrenergic inputs to brain centers including cortex, hypothalamus, and amygdala. Out of these neuroanatomical connections, the LC is innervated by the amygdala and hypothalamus via CRF neurons, thus forming a feed forward circuit [11, 12, 89]. During repeated stress this feed forward influence might be responsible for prolonged heightened neuronal firing in LC. The resilience or vulnerability of this feed-

forward system towards adjustment by allostasis might result in either the beneficial or detrimental effects of stress.

Administration of CRF_1 antagonists before inescapable shock prevents the development of LH but fails to do the same if the CRF_1 antagonist is given before escape testing [90]. Further association of LH behavior with LC comes from evidence wherein administration of α_{2A}-AR agonists into the LC reverses the behavioral effects of inescapable stress. α_{2A}-AR in LC inhibits firing upon activation by NE. Furthermore, intra-LC administration of α_{2A}-AR antagonists produces behavioral effects similar to those of inescapable stress [91-93]. Besides LC, another brain stem nucleus that is central to helplessness is dorsal raphe nucleus (DRN). Hyper-activation of 5-HT neurons in DRN is a key during uncontrollable stress-induced LH behavior, and increased LC firing might also be involved in DRN hyperactivity [23, 94-96]. DRN 5-HT neuronal activation induces, while lesions to DRN blocked, LH behavior [97, 98]. Further, LC derived NE stimulates α_1-AR in the DRN, causing hyper-activation of serotonin neurons. Intra-DRN administration of α_1-AR antagonist benoxathian prevented the behavioral effects of inescapable tail shock stress [23, 94-96] . DRN's direct role in LH development cannot be denied but LC can influence DRN activity via its noradrenergic projections and can make a contribution to LH behavior at the level of DRN also.

2.11 Amygdala and learned helplessness

Maier and colleagues has shown in a series of experiments that shuttle box escape deficits induced by uncontrollable stress can be independent of fear. Interventions that have the capacity to eliminate fear during escape testing, such as benzodiazepines or electrolytic lesions of the amygdala do not interfere with escape latencies/failures 24 h following TSS. One can postulate from the data that under conditions which do not allow transfer of fear from the initial stress condition to escape testing environment, the escape deficits are independent of fear [31, 87, 99]. Rats exposed to uncontrollable shocks in shuttle box (by feet or by electrodes attached to tail of rat place in shuttle box) exhibit escape deficit until 42 days post-stress [85, 100]. These escape deficits are dependent on conditioned fear and involve the basolateral amygdala, as lesions in this region reverse the escape deficit [86].

2.12 Perspective

Escape deficits observed after stress and escape testing in a similar context, or in distinctly different contexts, are both termed learned helplessness. However these escape deficits appears to be very different mechanistically. Therefore the LH model should be applied with caution when studying stress susceptibility and the drugs to treat stress-induced affective disorders such as depression. In the variation of model wherein context similarity between initial

stress and subsequent escape testing is employed, the observed prolonged escape deficits are confounded with contextual fear. Drugs that eliminate fear also can reverse escape deficit in this variation of LH model. This is a false positive as it is not reflective of an effective antidepressant action. Interpretations and inferences made from the results should always take into consideration the version of LH model employed. An important question that remains to be answered is, can a prolonged LH behavior be induced by applying inescapable stress in an environment different from the escape testing environment?

3. METHODS

3.1 Animals

Adult male Sprague–Dawley rats from Harlan Industries (Indianapolis, IN, USA) were acclimatized for 1–2 weeks before starting the behavioral testing procedures. Rats used were 250–350 g and had free access to food and water. Rats were housed in humidity and temperature-controlled room (23°C) on a 12-h light/dark cycle (lights on at 7 A.M). All the rats were handled for 15 minutes daily for 4 days before starting the experimental protocols. During the handling phase, all rats were transported in their home cages from the animal house to the behavioral core facility to acclimate the rats to transportation stress. All animal-related procedures were approved by the Creighton University Institutional Animal Care and Use Committee/University of Houston Institutional Animal Care and Use Committee and were performed in accord with the National Academy of Sciences Guide for the Care and Use of Laboratory Animals (7th Edition, 1996).

3.2 Learned helplessness model

3.2.1 Inescapable shock - Single stress studies

Rats were subjected to an inescapable shock session using a restrainer. The session consisted of 100 random un-signaled tail shocks from a constant-

current (DC) shock generator. The duration of each shock was 5 s, applied randomly at a 25-110 s inter-trial periods (mean inter-trial period was 60s) for approximately 2 hr. The inescapable tail shock started at 1.2mA and was increased by 0.2mA/20 shocks to 2mA. Delivery of the shocks was controlled by software from Med Associates (SOF-735: MED-PC® IV software, Med Associates Inc. St. Albans, VT). After the shock session, rats were returned to their home cages. The rats were initially placed in 2 groups; a tested control (TC) group, which was restrained with electrodes attached to the tail but not shocked, and a stressed group (inescapable, unpredictable tail shocks). There were four sets of experiments involving rats being euthanized 1h , 24h, 48h and 6 day(144h) post-stress **(Fig 5)** with or without single stress exposure. Brains were removed at these times and stored at -80^{o}C till cryosectioning.

3.2.2 Inescapable shock – Repeated stress studies

Similar to single stress, rats were exposed to inescapable tail shock (100 unpredictable shocks 1.2-2.0mA /↑0.2mA per 20 shocks) 3 times, on days 1, 4, and 7. After each shock session, rats were returned to their home cages. Initially, the rats were randomly distributed into 3 groups. In addition to repeatedly stress group and a TC group, a second control group, a naïve control (NC- not restrained, not shocked, handled similarly as TC and repeated stress rats) group was included. This group was added since the TC group was restrained 3 times

and it was possible that this might be stressful enough to induce behavioral and biochemical changes. The NC group enabled us to control for this possibility. Rats were euthanized on day 23 post-stress (**Fig 6)** in all groups; brains were removed and stored at -80^{o}C till cryosectioning.

3.3 Escape behavior testing

A shuttle box was used to create an escape task to assess responses of all rats. A plastic divider with a door separated the two chambers of the box with a plastic divider enabling escape movement for rats to terminate delivery of shocks. Before the test, each rat was acclimated to the box for 2 min. The first 5 fixed ratio-1 (FR1) trials required a single crossing from one side of the box to the other to terminate shock. This was to assess normal motor functioning response to foot shock. After the final FR-1 trial, rats did not receive further shocks for 5 min. The subsequent 25 fixed ratio-2 (FR-2) trials required two crossings from one side of the shuttle box and back to terminate the foot shock. For both FR-1 and FR-2 trials, a foot shock of 0.6 mA was randomly applied an inter-trial interval of 30- 90 s (mean inter-trial period was 60 s) using software from Med Associates. The time taken to perform the task required to terminate the shock during each trial (single, FR-1, or double crossing, FR-2) was the escape latency.

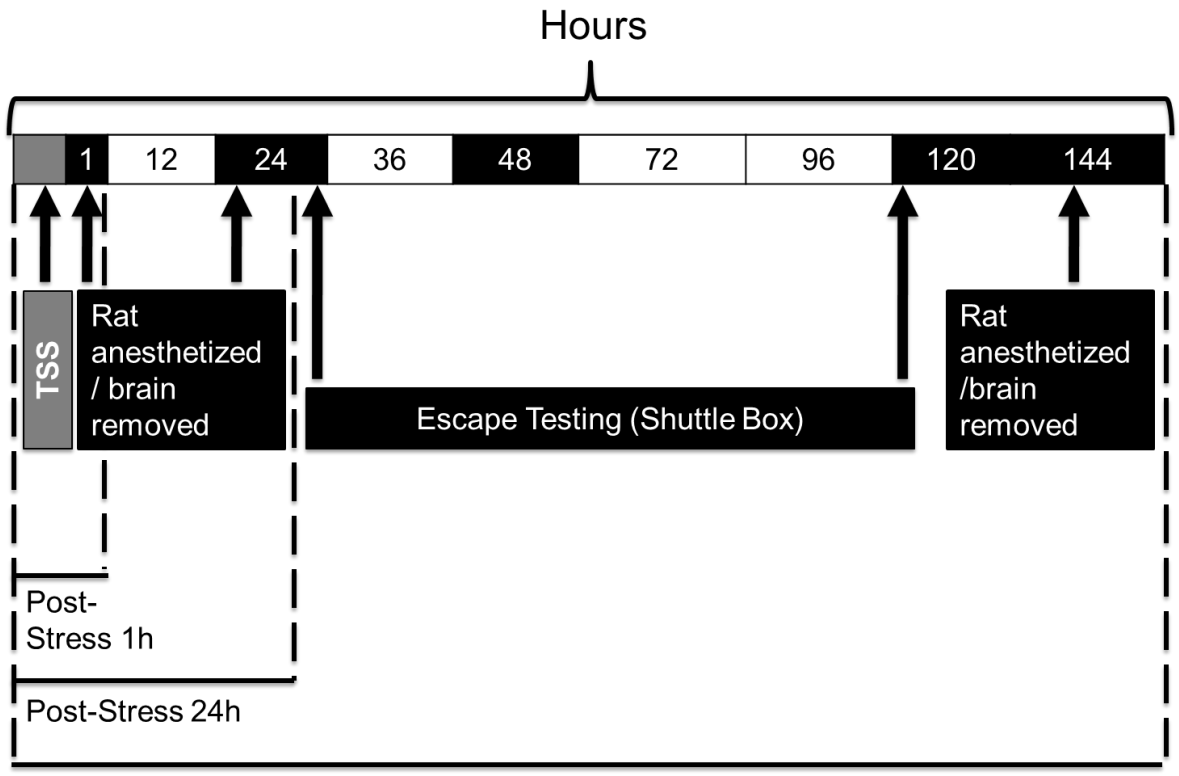


Figure 5: <u>Time line of different sets of single stress experiments</u>

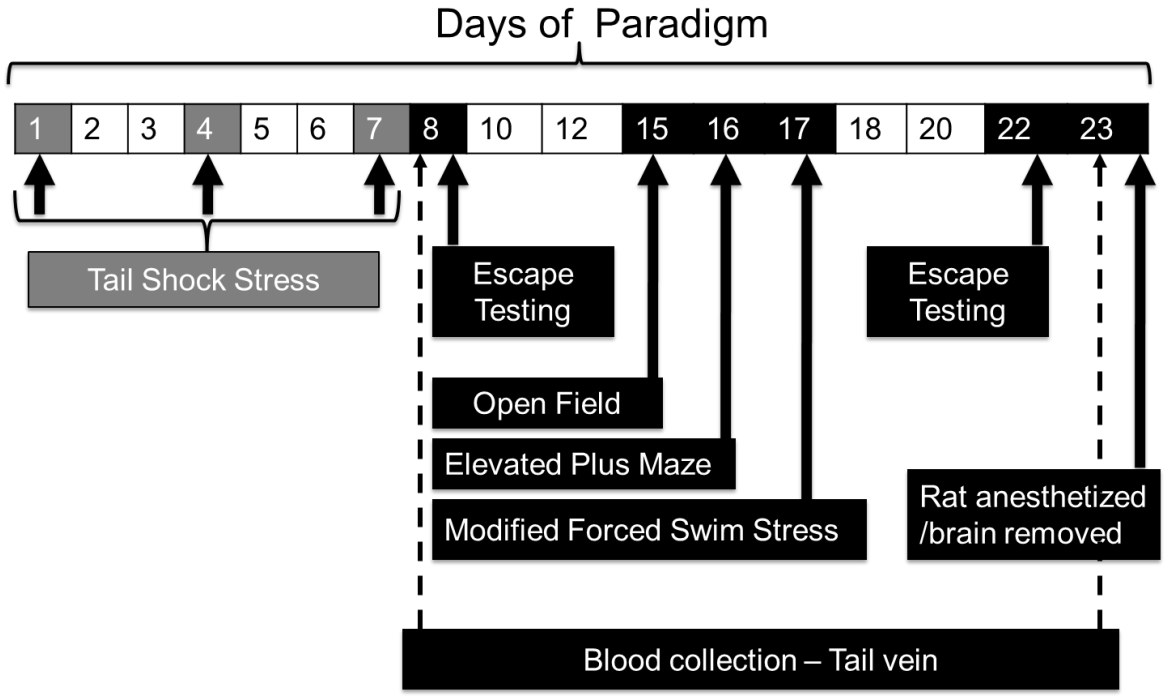


Figure 6: <u>Time line of events for repeated stress-induced sustained learned helplessness behavior</u>

If the rat did not terminate the shock within 30 s for any trial, the shock was automatically terminated and an escape latency of 30 s was recorded for that trial. When a rat is unable to complete an escape task in 30s, that particular trial is considered as an escape failure. Average escape latency and percentage (%) escape failures in 25 FR2 trials are considered indices of escape behavior.

In the single stress studies, the escape task (shuttle box test) is done 24h post-stress to identify learned helpless (stress susceptible) and non–helpless (stress-resilient) populations amongst stressed rats. To assess the disappearance of LH behavior after single stress, escape testing was done 120h (day 5) post-stress **(Fig 5)**. In the repeated stress studies escape testing is done on day 8 post-stress (24h after the 3rd bout of stress on day 7) to identify the learned helpless (stress susceptible) and non–helpless (stress-resilient) populations amongst the stressed rats. To demonstrate the sustainability of LH behavior, escape testing was done on day 22 post-stress (giving a window of 14 days of LH behavior) **(Fig 6)**.

3.4 Data analysis for learned helplessness

Escape latency times (s) / escape failures (%) in the FR-2 trials for each rat in single and repeated stress studies were averaged to determine the mean escape latency/failure of each rat. Single and repeated stress rats escape behavior data was then subjected to K-means cluster analysis. The analysis

identified two clusters within the stressed group. These clusters were compared by t-test. If the two clusters within the stressed group were statistically different, the cluster with the larger escape latency /greater % escape failure (index of escape deficits) was tentatively labeled LH while the other cluster was labeled NH. The labeling of LH and NH clusters was confirmed by comparing the escape latencies/% escape failure to those of control group (ANOVA followed by tukey test). If the escape latency/% escape failure of the LH cluster were significantly greater than TC, the LH label was confirmed. If the escape latency/% escape failure of the NH cluster were not different from TC, the NH label was confirmed.

Single stress: Disappearance of LH behavior was identified by:

1) 120h-post stress escape latency times / failures of singly stressed rats subjected to K-means cluster analysis failed to identify two statistically different clusters in singly stressed group.
2) LH rats identified 24h post-stress showing significantly lower escape latencies/failures 120h post-stress.

Repeated stress: Prolonged LH and NH behavior identified by:

1) Day 22 post-stress escape latency times / failures of repeatedly stressed rats subjected to K-means cluster analysis, showing significantly different clusters as was identified on day 8 post-stress.

2) Same LH and NH rats identified on the basis of day 22 post-stress escape latencies/failures as identified by day 8 post-stress escape latencies/failures.

3.5 Behavioral tests for cross validation of repeated stress studies

3.5.1 Open field test

The open-field test was carried out in plexiglas chambers (60 cm X 40 cm X 50 cm) in the behavioral core room with light intensity of 150 lux. Rats were placed at the center of the chamber and were allowed to explore the chamber for 30 min. A software driven Opto-Varimex Micro Activity Meter v2.00 system (Optomax, Columbus Instruments; OH) was used to gather and quantitate data comprising of rat movements and activities in the chamber. The chamber is fitted with sensors consisting of eight infrared light emitting diodes and eight photo transistors that emit and detect modulated infrared light beams respectively. The beams form two-dimensional cages and rearing can be monitored in each dimension. Movement of each rat is detected by beam breaks and recorded simultaneously. Data for each rat was collected in 3 min bins over a 30-min test session. Locomotor activity was analyzed using activity counts (total, vertical stereo and ambulatory activity). Exploratory activity was analyzed using counts for rotations, rearing, and stereotypic bursts. An approximately 25 cm × 25 cm

square in the center of the open-field arena was defined as the center zone for data analysis. The % total time spent, % total number of entries, and % total distance travelled in the center zone was used as an index for anxiety behavior (lower % indicative of anxiety trait). At the end of the 30-min test, the rat is removed from the plexiglas chamber and placed into its home page on a cart outside the behavioral testing room. The chamber was cleaned of any fecal material or urine. It was wiped thoroughly with 70% alcohol followed by a spray of water and dried with paper towels before (wait for 5-10 minutes to let off the aroma) another rat was tested.

3.5.2 Elevated plus maze test

The maze consisted of four arms (50 X 10 cm) (two open without walls and two enclosed by 30 cm high walls). The arms are elevated 50 cm off a base. The elevated plus maze has similar levels of illumination (30 lux) on both open and closed arms. Ethovision software (Noldus Information Technology, Asheville, NC) coupled with a digital video camera mounted overhead on the ceiling was used as a video-tracking system which automatically detects and records the movement of each rat. The video-tracking system (5 min trial) is started after the rat is placed at the center of the maze facing an open arm. The system will automatically record the number of entries made and time spent by the rat on the open and closed arms along with the central area. An arm entry is counted when

all four paws of the rat are on that arm. At the end of the 5-min trial, the rat is placed back inside its home cage. The elevated plus maze was cleaned of any fecal material and urine. It was wiped thoroughly with 70% alcohol followed by spray of water and dried with paper towels before another rat (wait for 3-5 minutes to let off the aroma) was tested.

3.5.3 Modified forced swim stress test

Rats were placed in a plexiglas cylinder (Lafayette Instrument Company Lafayette, IN) (21 cm diameter, 45 cm height) filled with 30 cm water (room temperature ~25°C). The water level was such that the rats with their tail were unable to touch the bottom of the cylinder. They were then subjected to a 15-min swim and videotaped by digital camera for subsequent scoring of their behaviors. Rats were immediately removed after the test, gently dried with a towel and placed in a cage with an overhead heat lamp until completely dry. The rats were then returned to their home cages. The plexiglas cylinder was attached to a PVC tubing to drain the water after each rat was tested. The water in the cylinder is changed for each rat. For behavioral scoring, the 15-min test was divided into 5-s bins, and scored for the predominant behavior for each bin: immobility, swimming, or climbing. The total number of scoring counts for each behavior for each rat was determined, a mean determined for each group and compared

among groups. The comparisons were done for 0-5 min, 5-10 min, 10-15 min and the total 15 min test period.

The three behaviors were defined by the following characteristics [101]:

Immobility: Minimal or no limb movement with horizontal, motionless, floating in order to maintain a steady non-traversing position on the surface of the water.

Swimming: Paddling movement of both the front and hind legs with a horizontal body position traversing across the surface of the water

Climbing: Vigorous kicking with hind legs below the surface of water accompanied by forepaws ambulating against the sides of the cylinder in order to maintain vertical position above the surface of water.

3.6 Chronic desipramine treatment: Reversal of prolonged LH behavior

Desipramine hydrochloride (Sigma Aldrich, St. Louis, MI, USA), was dissolved in 0.9% physiological saline. Since the drug has low solubility, it was dissolved by warming the solution. Drug solution was prepared daily and then was sterile filtered. 5.7mg/ml of desipramine hydrochloride (≈5mg/ml desipramine free base) was prepared and injected intraperitoneally (0.1ml/100g of body weight of rat) twice a day (7.30 am, 6.30 pm). The dosage of desipramine was extrapolated from the minimal clinically effective human dose of 100mg daily. The dose regimen started after the first escape test on day 8 (24h after

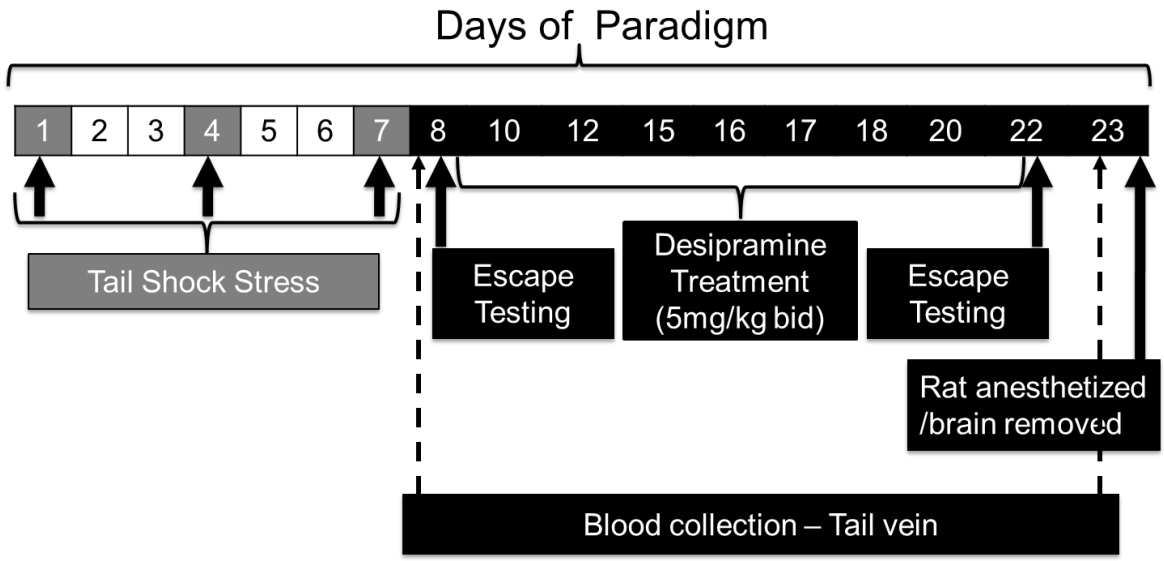


Figure 7: <u>Time line of events for chronic desipramine treatment within the repeated stress study</u>

3rd bout of stress-*repeated stress*) and continued till day 22 post–stress before the second escape test (total of 26 doses in 14 days) **(Fig 7)**.

3.7 Processing of plasma samples

Blood (≈0.5ml) was collected from the tail vein **(Fig 6, 7)** in Na_2EDTA coated micro vacutainers. Plasma was separated by centrifugation (1400rpm/10min/4° C). Plasma was divided into two aliquots (≈200µL and 50µL). 200µL of plasma sample was mixed with 8µL of a stabilizing solution (5g Na_2SO_3+3.5g EDTA per 100mL water) to prevent catecholamine oxidation. The 208µL (EPI and NE determination) and 50µL (Corticotrophin releasing factor and corticosterone determination) aliquots were stored at -80° C until analysis.

3.8 Estimation of stress hormones

3.8.1 Norepinephrine, Epinephrine

The stabilized plasma was extracted with 10mg Al_2O_3 (alumina) in 1.5M Tris HCl (pH 8.6) containing 1% Na_2EDTA and 10ng of an internal standard epinine was added. The samples were vortexed for 10 min, followed by centrifugation (10,000rpm/1min/4°C).The supernatant was discarded and sample adsorbed on alumina was washed with water (1ml twice, and centrifuged at 10,000rpm/1min/4° C after each wash). Perchloric acid (0.2N, 100µL) was mixed

thoroughly with washed alumina for catecholamine extraction. The supernatant containing the perchlorate extract after centrifugation (10,000rpm/1min/4° C) was filtered through a 4-mm nylon syringe filter (pore size of 0.45 μM) (National Scientific, Rockwood, TNA). Fifty μL of filtrate was injected into a high-performance liquid chromatography (HPLC) apparatus (Model 1525; Waters Corporation, Milford, MA)

Separation of NE and EPI were carried out at room temperature using C_{18} reverse phase 3μL LUNA column (100 X2.0 mm; Phenomenex, Torrance, CA,). The mobile phase composition was NaH_2PO_4(25 mm), Na-citrate (50 mm), EDTA (0.03 mm), diethylamine HCl(10 mm), and sodium octyl sulfate (2.2 mm) at a pH of 3.2, with Methanol (30 mL/L) and dimethylacetamide (22 mL/L). Mobile phase was degassed and filtered by passing through 0.45μm filter. HPLC analysis was done by isocratic elution of 50 μL of sample with a flow rate of 0.4 ml/min. The chromatographed peaks were detected by coulometric electrochemical detector (Model Coulochem III; ESA Inc., Chelmsford, MA, USA). The chromatographic run time was 10 min with the retention time for analyte NE-2.17 min, EPI-2.65 min, and, for internal standard epinine was 6.5min. Standard curve: (0.01–1 pg/μL of each analyte-NE and EPI) was constructed under the similar processing conditions during each run. Unknown sample concentrations were calculated by extrapolating the analyte peak area/Internal standard area ratio from the standard curve.

3.8.2 Corticotrophin releasing factor (CRF), corticosterone

CRF and corticosterone were estimated in plasma using an extraction free enzyme immunoassay kit as per manufacturer protocol (CRF-Bachem Americas, Inc. Torrance, CA; Corticosterone-Cayman Chemical Company, Ann Arbor, MI)

3.9 Brain region isolation and preparation of homogenates

3.9.1 Locus Coeruleus (LC) and cerebellum

Isolated brains were equilibrated at -20° C in a cryostat (Leica CM 1850, Leica Microsystems Inc., Bannockburn, IL). The brain stem was removed by making a coronal cut at most 1.0 mm rostral to the cerebellum. The whole brain stem portion was embedded in optimal cutting temperature (OCT) compound (Tissue Tek, Sakura Finetek USA, Inc. Torrance, CA) at -20^{o}C to form a uniform block of OCT/tissue that was adhered to a mounting disc. A series of 300µm sections from the tissue block were generated and sections containing the LC were identified using the floor of the fourth ventricle as a reference. One mm^2 tissue plugs of LC and cerebellum were punched and transferred to eppendorf tubes containing 100µl freshly prepared homogenization buffer (400 µL of 25 X complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) + 100 µL of 100 mM PMSF solution + volume up to 10 mL with Tris Buffer- pH 7.4 (10mM Tris Base +0.25M sucrose +1% SDS + 1mM EDTA). Samples were

analyzed for presence of tyrosine hydroxylase (TH) protein and 4-fold enrichment of TH over cerebellum samples from the same slices confirmed LC sample localization. A plastic dounce homogenization pestle was used to homogenize the tissue manually and the homogenates were boiled at 100ºC for 10 min. After cooling, the homogenates were centrifuged at 10,000 rpm/4^{o}C for 10 min. Aliquots of the supernatant were taken to estimate protein content and the remaining sample was diluted with 4X sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 0.1 mg/ml bromophenol blue) and stored at -80^{o}C until analysis by Western Blot.

3.9.2 Cortex, amygdala, striatum, hypothalamus, and hippocampus

The remainder of each frozen brain was put on a dry ice-cooled petri dish with the ventral side up and a few drops of ice cold saline were dropped on the area of hypothalamus which was then removed by pinching with a curved set of forceps. Then a coronal cut at 0.75mm from the rostral end of the brain (ventral side down) was made to separate the cortex. A sagittal cut was made at 2.9 mm from midline followed by a second cut, an additional 1.5mm from midline. The two 1.4 mm slices from both hemispheres were separated. Hippocampus, amygdala, and striatum nuclei regions were isolated and removed from these slices. The isolated tissues were homogenized using freshly prepared homogenization buffer (300 µL for hippocampus/amygdala/striatum/

hypothalamus and 400µL for cortex). Tissues were homogenized (PRO200 post-mounted laboratory homogenizer; MIDSCI, St. Louis, MO) using a 5 x 75 mm (DXL) flat style probe (2 x 10 sec, 12000 rpm). After this step, homogenates were processed as described previously for LC samples.

3.10 Protein estimation

A Pierce protein detection kit (Pierce, Rockford, IL) containing BCA protein assay reagent A and reagent B was used to determine protein concentrations [102].

3.11 Western blot analysis

Homogenates diluted with 4x sample buffer were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and resolved proteins were electrophoretically transferred at 90V for 2.5h (4°C) to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare Biosciences, Piscataway, NJ). The PVDF membrane was then incubated for 1 h in 5% nonfat dry milk in TBS-T (20 mM Tris-HCl (pH 7.6), 137 mM NaCl and 0.1% Tween 20). Membranes were probed for G-protein coupled receptor kinase 3/2 (GRK3, GRK2), TH and loading control β-actin or glyceraldehye-3-phosphate dehydrogenase (GAPDH). After probing for GRK3 protein , the membranes were stripped (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 100 mM 2-mercaptoethanol) for 25 minutes and then washed in TBST for followed by blocking with 5% nonfat dry milk for 30 minutes. These stripped and

blocked membranes were reprobed for GRK2 protein. Similar stripping, blocking and immunoblotting procedure on the same membrane was done for loading control and H_1-R. The order of probing the proteins are (i) GRK3, (ii) GRK2, (iii)TH/GAPDH or TH/ β-actin (iv) H_1-R (repeated stress samples only). The membranes were incubated with anti- GRK3/ GRK2/H_1-receptor and anti-TH/GAPDH or β-actin for 1.5h **(Table 1)**. After washing the membranes were further incubated with an anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody (GRK3/2 andH_1-receptor) and anti-mouse HRP-linked secondary antibody (TH/β-actin or TH/GAPDH) for 1h **(Table 1)**. The images of immunoblots were captured by a fluorchem imaging system (Alpha Innotech Corp., San Leandro, CA). Intensity of each immunoreactive band was determined Alphaease 4.0 (Alpha Innotech Corp., San Leandro, CA) and was normalized to the GAPDH or β-actin loading control.

Another set of the same tissue lysates samples were subjected to the same procedure for western blot and probed for Alpha$_{2A}$-adrenoceptor (α_{2A}-AR). After stripping, the membranes were blocked and probed for corticotrophin releasing factor 1 receptor (CRF_1-R) protein and finally with anti-GAPDH or β-actin for loading control. The membrane was incubated with anti- α_{2A}-AR/ CRF_1-R overnight at 4^{o}C and anti- GAPDH or β-actin for 1h **(Table 1)**. After washing the membrane was incubated with an anti-goat HRP-linked secondary antibody (α_{2A}-AR/ CRF_1-R) and anti-mouse HRP-linked secondary antibody (GAPDH/ β-

actin) at room temperature for 1h **(Table 1)**. Images of blots were taken and processed as described above.

Primary Antibody	Dilution Used	Secondary Antibody	Dilution Used
Rabbit polyclonal anti- GRK3	1:500	Anti-rabbit HRP	1:500
Rabbit polyclonal anti- GRK2	1:1000	Anti-rabbit HRP	1:1000
Rabbit polyclonal anti- H_1-Receptor	1:500	Anti-rabbit HRP	1:500
Mouse monoclonal anti-TH	1:1000	Anti-mouse HRP	1:1000
Mouse monoclonal anti-GAPDH	1:1000	Anti-mouse HRP	1:1000
Mouse monoclonal anti-β-actin	1:1000	Anti-mouse HRP	1:1000
Goat polyclonal anti-α_{2A}-AR	1:300	Anti-goat HRP	1:500
Goat polyclonal anti- CRF_1-R	1:500	Anti-goat HRP	1:500

Table 1: <u>Dilutions of primary and secondary antibodies used for immunoblotting</u>
Anti-GRK3/GRK2/H_1-receptor, anti- α_{2A}-AR/ CRF_1-R, anti- β-actin, anti-rabbit, and anti-mouse antibodies were from Santa Cruz Biotechnology Inc. (Santacruz, CA). Anti-TH/GAPDH antibodies were from Millipore (Billerica, MA).

3.12 Data analysis

3.12.1 Western blots

Mean normalized intensities of immunoreactive bands were compared between groups by one-way ANOVA followed by Tukey's post hoc test or Student's t-test whichever is appropriate (Prism, Graph Pad Software, San Diego, CA), and groups were considered significantly different if $p \leq 0.05$. Data points were excluded when Grub's test indicated a statistical outlier at $p \leq 0.05$. Results are expressed as % change in Means ± S.E.M as compared to control.

3.12.2 Behavioral parameters/stress hormone levels

Means were compared between groups by one-way ANOVA followed by Tukey's post hoc test or Student's t-test whichever is appropriate (Prism, Graph Pad Software, San Diego, CA), and groups were considered significantly different if $p \leq 0.05$. Results are expressed as Means ± S.E.M.

3.12.3 Cluster analysis: Biochemical parameters in single stress studies

The biochemical (levels of proteins- GRK3, α_{2A}-AR, CRF_1) and behavioral (escape latencies) variables were used to independently identify two clusters using K-Means cluster analysis. ANOVA was performed on the TC group and the two identified clusters and the clusters were considered significantly different if

$p \leq 0.05$. The clusters identified on the basis of biochemical parameters alone were then compared with clusters identified by the behavioral parameter to evaluate the accuracy of the biochemical estimations (GRK3, α_{2A}-AR, CRF_1-R) in predicting the behavior of stressed animals. This whole procedure was done for LC, amygdala, and cortex. Cluster analysis was performed using SAS 9.2 (SAS Institute Inc., Cary, NC). In the experiment wherein rats were sacrificed 1h or 24 h post-stress without escape behavior testing, cluster analysis of biochemical data was used to identify the existence of two groups among stressed rats. Levels of GRK3, α_{2A}-AR, and CRF_1 were used as variables to identify clusters from the groups of stressed rats without escape behavior testing.

4. RESULTS

4.1 Single stress-induced learned helpless (LH) and non-helpless (NH) behavior- 48h- post-stress

4.1.1 Behavioral segregation of rats subjected to single stress paradigm

Stressed rats showed higher escape latencies and escape failures (an index of escape deficits) compared to TC rats (**Fig 8A, B)** in FR2 trials. However when escape latency and escape failures data for the stressed rats were subjected to K-means cluster analysis, the data showed a bimodal distribution. This bimodal distribution suggested the presence of two different populations within the group of stressed rats **(Fig 8C, D)**. One population exhibited mean escape latencies and escape failures significantly larger than the TC group, and was designated as learned helpless (LH) rats. The second population did not differ from the TC group, and was designated as non-helpless (NH) rats.

The LH and NH populations are approximately equally (50%) distributed within the stressed rats. 5 FR1 trial with an easier escape task showed that there was no difference across the TC, LH, and NH populations, suggesting that locomotor deficits in LH rats are not responsible for the higher escape deficits in the FR2 trials.

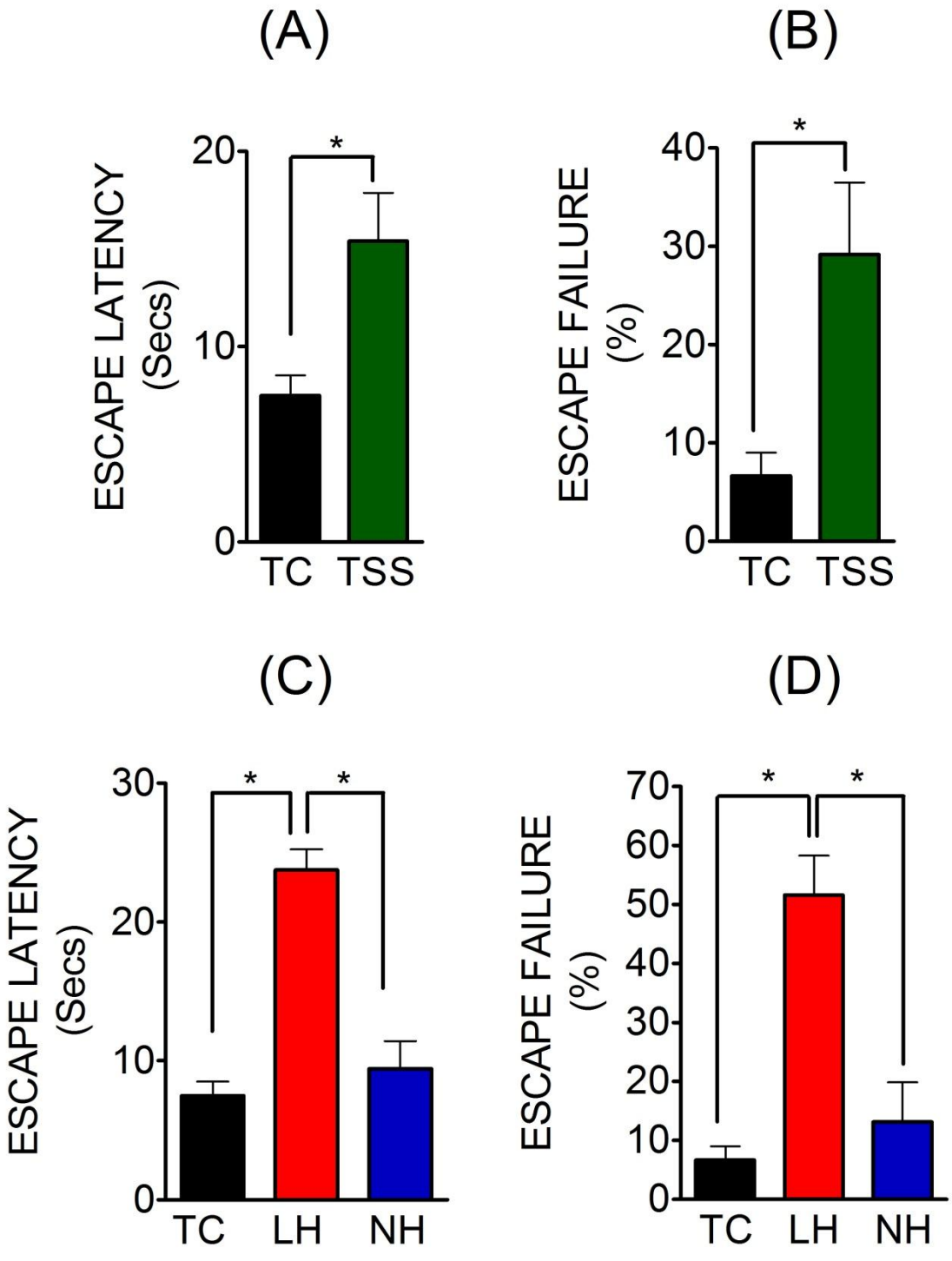

(A)
ESCAPE LATENCY
(Secs)
20
10
0
*
TC
TSS
(B)
ESCAPE FAILURE
(%)
40
30
20
10
0
*
TC
TSS
(C)
ESCAPE LATENCY
(Secs)
30
20
10
0
*
*
TC
LH
NH
(D)
ESCAPE FAILURE
(%)
70
60
50
40
30
20
10
0
*
*
TC
LH
NH

Figure 8 : <u>Escape behavior data of single stress rats reveals two distinct behaviors</u>

Rats were randomly separated into two groups. One group, the TC, was restrained but did not receive single bout of tail shock stress (TSS). The other group received single stress (unpredictable, inescapable 100 tail shocks/ 1.2-2.0mA increased by 0.2mA/20shocks). Twenty four hour after stress or after restraint for TC, escape latencies and escape failures were determined by escape testing. Escape behavior data of stressed rats was subjected to K-means cluster analysis, which identified the presence of two populations within a group of stressed rats (LH-higher, NH-similar escape latencies/escape failures to TC).

Panel (A) Comparison of mean escape latencies of TC and single stress rats.
Panel (B) Comparison of mean escape failures of TC and single stress rats.
Panel (C, D) Escape latencies and escape failures of LH and NH rats identified by cluster analysis; the presence of two populations was confirmed by comparison with TC rats. Single stressed rats (n=12) rats showed a bimodal distribution of 5 LH rats (greater escape latencies/failures than TC rats) and 7 NH rats (escape latencies/failures similar to TC rats). Data is expressed as Mean ± SEM, and * - mean escape latency/failure significantly different (LH vs. NH) and (LH vs.TC) ($p \leq 0.05$) (n=5-12).

4.1.2 Biochemical assessment of signaling molecules

4.1.2.1 Locus Coeruleus (LC)

Exposure to a single bout of stress resulted in a specific reduction of GRK3 associated with LH behavior and down-regulation of α_{2A}-AR and CRF_1-R associated with NH behavior. In contrast to GRK3, GRK2 levels in LC were unchanged across the groups. GRK3 levels were reduced in LC of LH rats (-28.1%; $p \leq 0.05$) compared to TC **(Fig 9E)**. These changes were specific to the LH group of stressed rats, because GRK3 was unchanged in NH group (-6.9%). α_{2A}-AR levels were down-regulated in the LC of NH rats (-46.3%; $p \leq 0.05$) compared to TC **(Fig 9E)**, but α_{2A}-AR levels in LH rats were unchanged (-6.5%). Similar to the α_{2A}-AR, single stress produced behaviorally specific changes in CRF_1-R levels, which were down-regulated in the NH rats (-32.2%; $p \leq 0.05$) but were unchanged in LH rats **(Fig 9E)**. This suggests that single stress results in an adaptive down-regulation of α_{2A}-AR and CRF_1-R in NH rats that is absent in LH rats (again a behavior specific change). The lack of receptor down-regulation in LH rats correlates with reduction of GRK3 levels in same rats.

Tyrosine hydroxylase (TH) was used in these studies as a marker for catecholamine-containing neurons and particularly to indicate the concentrated presence of noradrenergic neurons in LC. In TC rats, TH levels in LC samples were 4-fold higher than in cerebellar samples punched from the same slice. TH

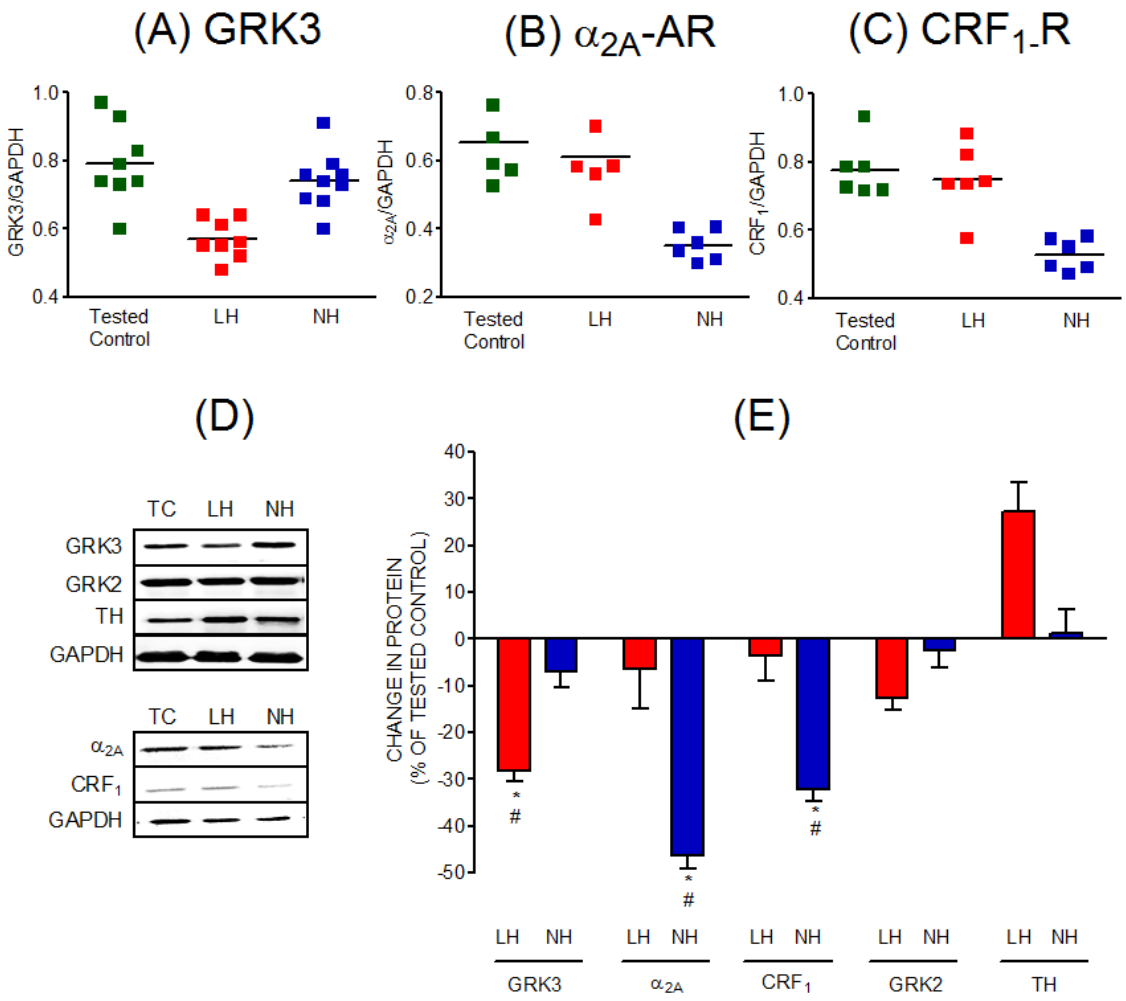

(A) GRK3
(B) α2A-AR
(C) CRF1-R
GRK3/GAPDH
α2A/GAPDH
CRF1/GAPDH
Tested Control
LH
NH
(D)
TC
LH
NH
GRK3
GRK2
TH
GAPDH
α2A
CRF1
(E)
CHANGE IN PROTEIN (% OF TESTED CONTROL)
GRK3
α2A
CRF1
GRK2
TH

Figure 9: <u>Changes in GRK3, α_{2A}-AR, and CRF_1 levels in locus coeruleus-48h post-stress (single stress)</u>

Rats were randomly separated into two groups. One group, the TC, was restrained but did not receive single bout of stress. The other group received single bout of stress (unpredictable, inescapable 100 tail shocks/ 1.2-2.0mA increased by 0.2mA/20shocks). Twenty four hour after single stress , escape latencies and escape failures data of stressed rats was subjected to K-means cluster analysis, to identify the presence of two populations within stressed rats (LH-higher, NH-similar escape latencies and escape failures to TC). Forty eight hour post-stress (24h after escape testing), rats were euthanized and brains were removed. LC was isolated from brains of TC, LH and NH rats and levels of GRK3, GRK2, TH , α_{2A}-AR and CRF_1-R were measured using western blot technique. Mean immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R, GRK2, TH /Loading control) ratios were calculated followed by change in protein for LH, NH rats alone are calculated in comparison to tested control.

Panel (A, B, C) Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios of LH, NH rats in comparison to tested control. **Panel (D)** Representative western blots for GRK3, GRK2, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (E)** Change in protein for LH and NH rats expressed as % of tested control. Significant change in protein levels ($p \leq 0.05$) *- (LH vs.TC), and # - (LH vs. NH) n=6-9.

levels were increased by single stress in LC (+27.2%- though not significant) of LH, but not NH, rats **(Fig 9E)**.

4.1.2.2 Amygdala

Similar to LC, exposure to single bout of stress resulted in a specific reduction of GRK3 associated with LH behavior. Relatively lower levels of α_{2A}-AR and CRF_1-R were associated with NH behavior. GRK3 levels were reduced in LH rats (-21.0%; $p \leq 0.05$) while there were no significant changes in NH rats (-7.1%) compared to TC (**Fig 10E)**. By contrast, single stress resulted in up-regulation of α_{2A}-AR in the amygdala of LH rats (+48.6%; $p \leq 0.05$). The up-regulation was greater in LH rats compared to NH rats (+25.2%) **(Fig 10E)**. CRF_1-R were down-regulated in NH rats (-32.2%; $p \leq 0.05$) but were unchanged in LH rats (-8.2%) compared to TC. **(Fig 10E)**. Similar to LC, amygdala also exhibited behavioral specific changes at the level of GRK3, α_{2A}-AR, and CRF_1-R proteins. TH and GRK2 levels were not appreciably altered in the amygdala of LH and NH rats.

4.1.2.3 Cortex

In contrast to LC and amygdala, single stress produced stress specific reduction of GRK3 in both LH and NH rats rather than behavior specific reductions. These reductions were associated with similar level of increase in α_{2A}-AR and decrease in CRF_1-R levels. GRK3 levels were reduced in both LH

and NH rats (-29.8% and -33.2%; p≤0.05) compared to TC, suggesting a change that was single stress-induced and unrelated to the behavioral consequence of the stress **(Fig 11E)**. GRK2 levels also were reduced in both LH (-16.4%; p≤0.05) and NH (-17.9%; p≤0.05) groups compared to TC **(Fig 11E)**, but these decreases were less than the changes in GRK3. Single stress resulted in up-regulation of α_{2A}-AR in the cortex. However, the increases in α_{2A}-AR levels were similar in the LH and NH rats (+28.8 vs. +34.0%) **(Fig 11E)**. Single stress also produced significant and comparable down-regulation of CRF_1 receptors in cortex compared to TC regardless of the behavioral effects of the stress (-32.6% vs. -30.2%; p≤0.05) **(Fig 11E)**. This suggests that GRK3 levels are not linked to changes in CRF_1-R in cortex, unlike the LC and amygdala. TH levels were not significantly altered in the cortex of LH and NH rats, probably due to lower and diffused noradrenergic neuronal abundance in this brain region.

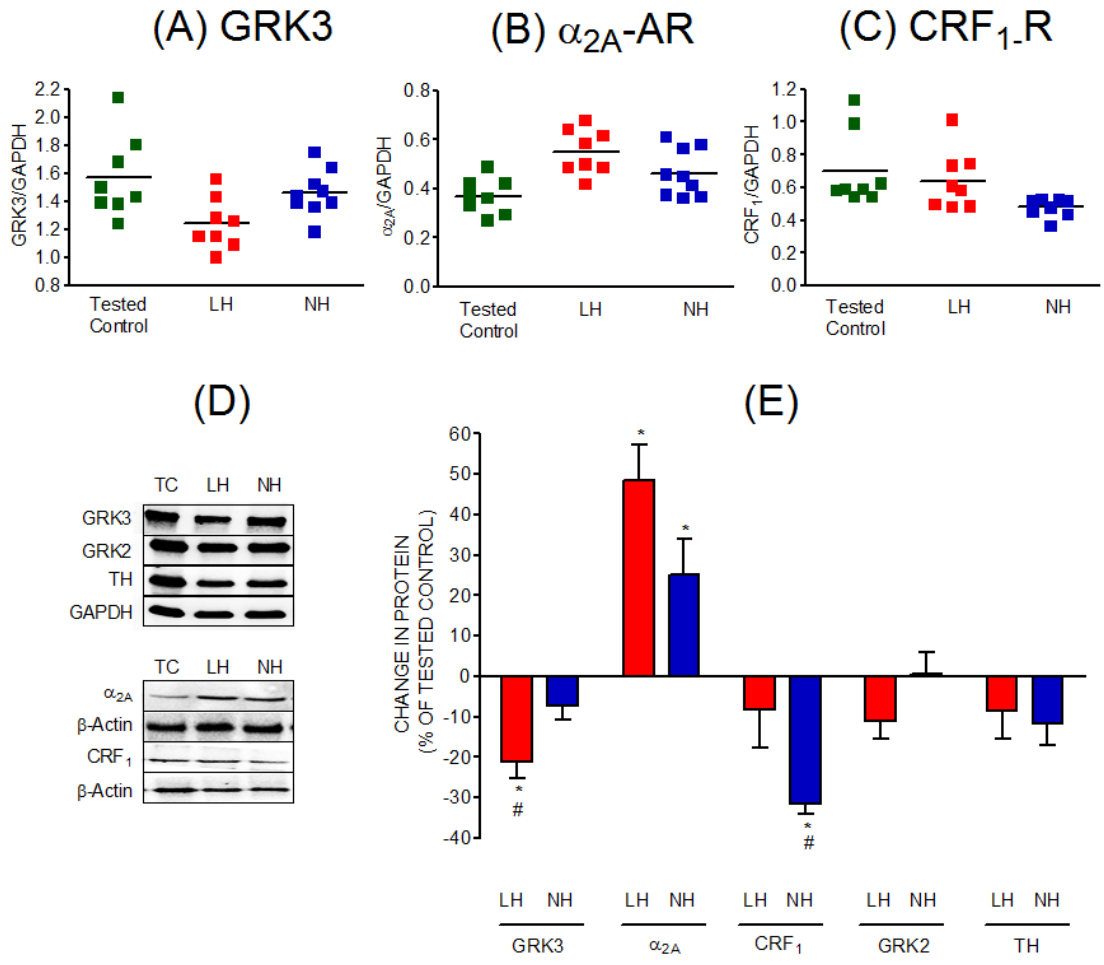

(A) GRK3
GRK3/GAPDH
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
Tested Control
LH
NH
(B) α2A-AR
α2A/GAPDH
0.8
0.6
0.4
0.2
0.0
Tested Control
LH
NH
(C) CRF1-R
CRF1/GAPDH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Tested Control
LH
NH
(D)
TC LH NH
GRK3
GRK2
TH
GAPDH
TC LH NH
α2A
β-Actin
CRF1
β-Actin
(E)
CHANGE IN PROTEIN (% OF TESTED CONTROL)
60
50
40
30
20
10
0
-10
-20
-30
-40
*
#
LH NH
GRK3
α2A
CRF1
GRK2
TH

Figure 10: <u>Changes in GRK3, α_{2A}-AR, and CRF_1 levels in amygdala 48h post-stress (single stress)</u>
Tested control (TC) and stressed rats, 48h post-stress (24h after escape testing), were euthanized and their brains were removed. Amygdala was isolated from brains of TC, LH and NH rats and levels of GRK3, GRK2, TH , α_{2A}-AR and CRF_1-R were measured using western blot technique **(For details refer to Fig 9 legend)**

Panel (A, B, C) Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios of LH, NH rats in comparison to TC. **Panel (D)** Representative western blots for GRK3, GRK2, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (E)** Change in protein for LH and NH rats expressed as % of tested control. Significant change in protein levels ($p \leq 0.05$) *- (LH vs.TC), and # - (LH vs. NH) n=8-9.

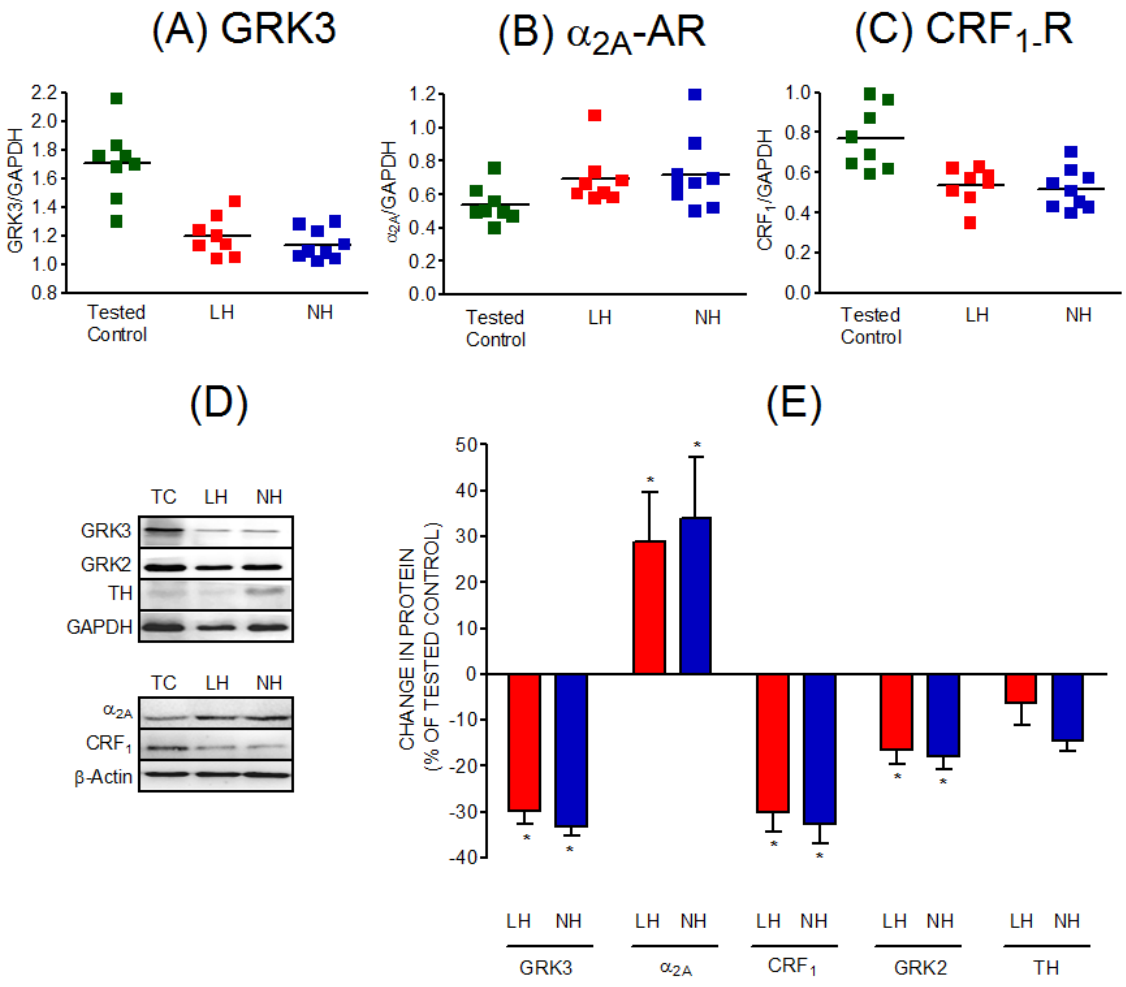

(A) GRK3
GRK3/GAPDH
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
Tested Control
LH
NH
(B) α2A-AR
α2A/GAPDH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Tested Control
LH
NH
(C) CRF1-R
CRF1/GAPDH
1.0
0.8
0.6
0.4
0.2
0.0
Tested Control
LH
NH
(D)
TC LH NH
GRK3
GRK2
TH
GAPDH
TC LH NH
α2A
CRF1
β-Actin
(E)
CHANGE IN PROTEIN (% OF TESTED CONTROL)
50
40
30
20
10
0
-10
-20
-30
-40
LH NH
GRK3
LH NH
α2A
LH NH
CRF1
LH NH
GRK2
LH NH
TH

Figure 11: Changes in GRK3, GRK2, α_{2A}-AR, and CRF_1 levels in cortex 48h post-stress (single stress)

Tested control (TC) and stressed rats, 48h post-stress (24h after escape testing), were euthanized and their brains were removed. Cortex was isolated from brains of TC, LH and NH rats and levels of GRK3, GRK2, TH , α_{2A}-AR and CRF_1-R were measured using western blot technique **(For details refer to Fig 9 legend)**

Panel (A, B, C) Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios of LH, NH rats in comparison to TC. **Panel (D)** Representative western blots for GRK3, GRK2, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (E)** Change in protein for LH and NH rats expressed as % of tested control. Significant change in protein levels ($p \leq 0.05$) *- (LH, NH vs.TC) n=8-9.

4.1.3 Changes in GRK3, α_2-AR, or CRF_1 -R levels are valid predictors of learned helpless behavior.

K-means cluster analysis using levels of GRK3, α_{2A}-AR, or CRF_1 receptor levels (48 h post stress-data) independently as individual variables was used to define the presence or absence of two clusters, if present, in LC, amygdala, and cortex of all single stressed rats. Then, identified clusters were compared by one-way ANOVA to each other and to the TC. Clusters identified were matched with NH and LH populations identified by independent K-means cluster analysis of escape testing of all single stressed rats. The percentage overlap of biochemically identified clusters with the stress resilient (NH) and stress susceptible (LH) populations identified by escape testing was used to evaluate the validity of the statistical model.

Analysis of GRK3 levels suggested two clusters in LC and amygdala of single stressed rats, one similar to TC and one with reduced GRK3 compared to TC. There was 94% and 82% overlap of these clusters with the LH and NH populations respectively **(Fig 12, Table 2, 3)**. In LC, all of the samples with reduced GRK3 levels were in the LH population **(Fig 12, Table 2)**. In amygdala, 6 out of 8 samples in the cluster with reduced GRK3 levels compared to TC were in the LH population **(Fig 12, Table 2, 3)**. In cortex, the GRK3 clusters identified were not significantly different from each other, although the stressed rats GRK3 levels were different from TC **(Fig 12, Table 2, 3)**.

Protein/Brain Region	Locus Coeruleus		Amygdala		Cortex	
	LH	NH	LH	NH	LH	NH
GRK3	-28.1*	-6.9	-21.0	-7.1	-29.8*	-33.2*
α_{2A}-AR	-6.5	-46.3*	48.6*	25.2*	28.8*	34.0*
CRF_1-R	-3.6	-32.2*	-8.2	-31.4*	-32.6*	-30.2*
GRK3/α_{2A}-AR/CRF_1-R changes linked to:	Stress Susceptibility		Stress Susceptibility		Stress	

Table 2: Changes in protein in locus coeruleus, amygdala, and cortex. 48h after single stress

Mean immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios of LH and NH rats obtained by western blotting are expressed as % of tested control (TC). Significant change in protein levels ($p \leq 0.05$) *- (LH, NH vs.TC)

Protein/Brain Region	Locus Coeruleus	Amygdala	Cortex
GRK3	94	82	59
α_{2A}-AR	92	71	53
CRF_1-R	92	77	53
GRK3+ α_{2A}-AR+ CRF_1-R	100	83	59

Table 3: % Overlap of clusters identified in locus coeruleus, amygdala and cortex by protein levels with NH and LH populations identified by escape behavior after single stress

GRK3, α_{2A}-AR, CRF_1-R levels were used independently to identify two clusters by K-means cluster analysis. % overlap of clusters was calculated by matching every observation of each cluster identified with protein levels to every observation of population identified by behavioral outcome.

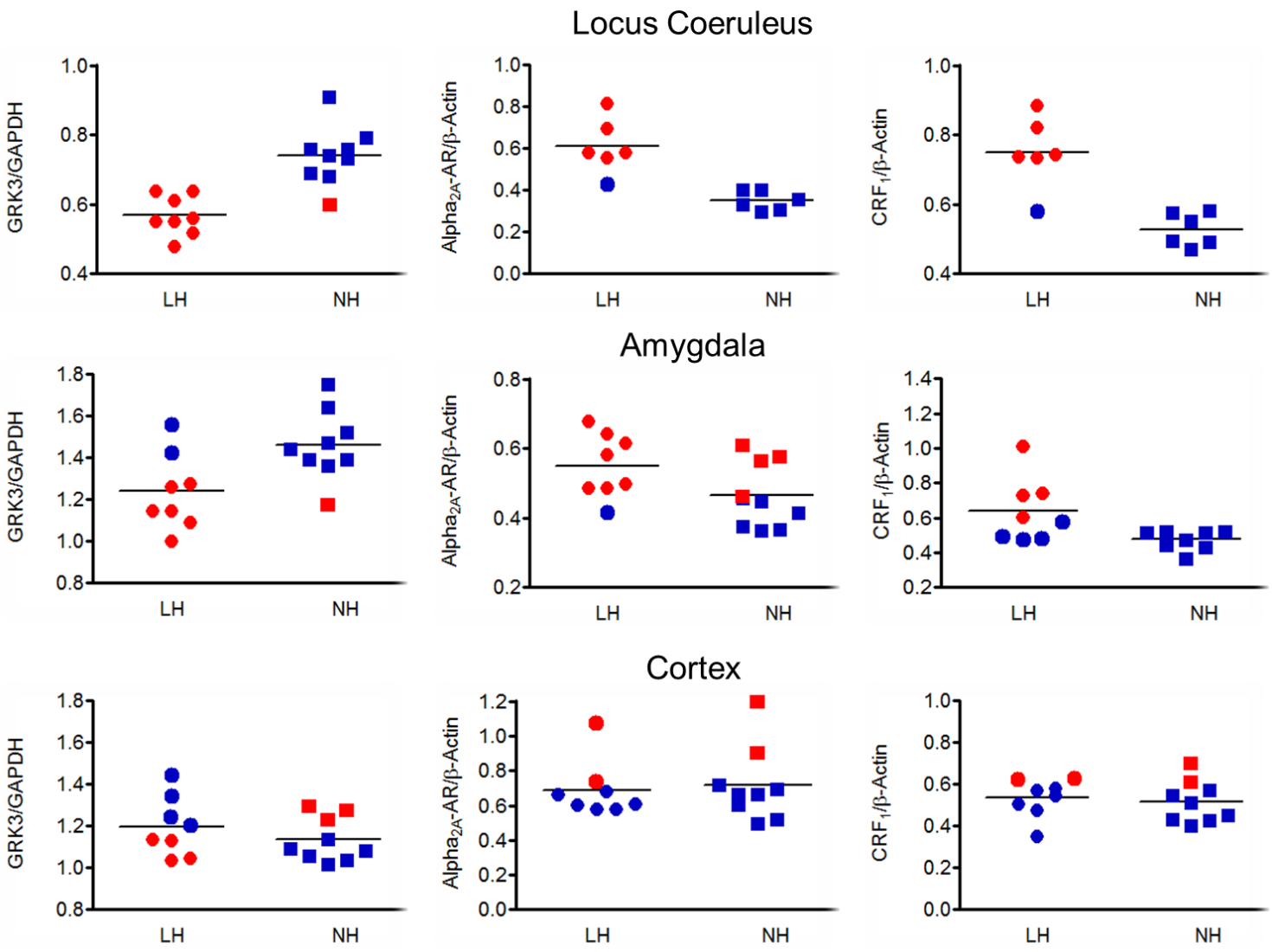

Locus Coeruleus
GRK3/GAPDH
Alpha2A-AR/β-Actin
CRF1/β-Actin
Amygdala
GRK3/GAPDH
Alpha2A-AR/β-Actin
CRF1/β-Actin
Cortex
GRK3/GAPDH
Alpha2A-AR/β-Actin
CRF1/β-Actin
LH
NH

Figure 12: Comparison of populations within the single stress group identified by cluster analysis of escape behavior versus clusters identified by protein levels

In each panel, circles represent data points from LH rats while squares represent data points from NH rats as determined by K- means cluster analysis of escape behavior data. Blue and red symbols represent the two clusters identified on the basis of the biochemical parameter represented in each panel.

Analysis of receptor levels revealed differences in the biochemistry versus the behavior associated with different brain regions. Cluster analysis of α_{2A}-AR levels in LC, amygdala, and cortex, revealed that in amygdala and cortex two statistically different clusters based upon α_{2A}-AR levels could not be identified. However in LC, one cluster based on α_{2A}-AR levels was similar to TC and the other was significantly lower than TC. Moreover, there was 92% overlap between these clusters in LC and the LH and NH populations identified by escape testing **(Fig 12)**. The NH population overlapped with the cluster with lower α_{2A}-AR levels while the LH population overlapped with the α_{2A}-AR cluster not different from TC. In both amygdala and cortex there were not two clusters of α_{2A}-AR levels in stressed rats but the α_2-AR levels in stressed rats were significantly higher than in TC (**Table 2, 3)**.

Analysis of CRF_1-R levels in LC and amygdala identified two significantly different clusters within the stressed rats. In both areas, one cluster had CRF_1-R levels similar to TC while the other cluster had levels significantly lower than TC **(Fig 12)**. The reduced CRF_1-R level clusters in LC and amygdala overlapped 100% and 74%, respectively, with the NH population. In cortex there were not two clusters of CRF_1-R levels in stressed rats but the CRF_1-R levels in stressed rats were significantly lower than in TC (**Table 2, 3)**. In amygdala, 82, 71 and 77% agreement was observed between the clusters identified by cluster analysis for GRK3, α_2-AR and CRF_1-R respectively, and the LH and NH populations. This

overlap is considerably lower than the 94, 92, and 92% agreement for GRK3, α_2-AR and CRF_1-R, respectively, that we observed in LC. However, when all GRK3, α_{2A}-AR and CRF_1 levels were used collectively as predictor variable in our statistical model, the accuracy in predicting behavior was 100% in LC and 83% in amygdala **(Table 3)**.

4.2 Effects of single stress on GRK3, α_{2A}-AR, and CRF_1-R levels are similar with or without behavioral testing

Rats were subjected to a single bout of tail shock stress and 24h later, at the time escape testing had been performed in previous groups, brains were harvested and protein levels were measured in LC, amygdala, and cortex. The cluster analysis statistical model using GRK3, α_{2A}-AR and CRF_1-R levels collectively as predictor variables in identifying LH and NH population independent of behavioral testing had shown 100 and 83 % accuracy. Therefore this statistical model was utilized to identify two groups on the basis of GRK3, α_2-AR and CRF_1 levels collectively in LC and amygdala, at the 24h time point where rats were stressed but not tested for escape behavior.

4.2.1 Segregation into two groups by cluster analysis: LC 24h post-stress

Cluster analysis in LC based on GRK3, α_{2A}-AR and CRF_1-R levels revealed the existence of two significantly different clusters in LC 24h post-stress

and prior to escape testing. In LC, when all single stressed rats were compared to TC, there were no significant differences in GRK3, α_{2A}-AR, CRF_1-R, GRK2 or TH levels **(Fig 13E)**. However, when the data for GRK3, α_{2A}-AR, CRF_1-R collectively for each rat were subjected to K-means cluster analysis, two clusters were identified, cluster C1 having n=12 (57%) and cluster C2 having n=9(43%) out of a total 21 stressed rats **(Fig 13B, C, F)**. The percentage of rats in each identified cluster was similar to the groups identified by behavioral analysis as LH and NH.

After cluster analysis, the data for each variable (GRK3, α_{2A}-AR, and CRF_1-R) were grouped according to the identified clusters (C1, C2 significantly different from each other for each GRK3, α_{2A}-AR, CRF_1-R levels) and compared to the TC group for each variable. GRK3 levels in cluster C1 were slightly higher that TC while GRK3 levels in cluster C2 were decreased (-36.6%; $p \leq 0.05$) relative to TC **(Fig 13B, F)**. Cluster C1, associated with a slight increase in GRK3, was characterized by a decrease in α_{2A}-AR (-24.7%) and CRF_1-R (-25.8%; $p \leq 0.05$) levels when compared to TC **(Fig 13F)**. Cluster C2, associated with a decrease in GRK3, was characterized by an increase in α_{2A}-AR (+44.5%; $p \leq 0.05$) and CRF_1-R levels (+12.5%) when compared to TC **(Fig 13F)**. In contrast to GRK3, α_{2A}-AR or CRF_1-R, neither GRK2 nor TH were significantly different from TC, whether grouped as all stressed rats or segregated into the identified clusters 24h post-stress.

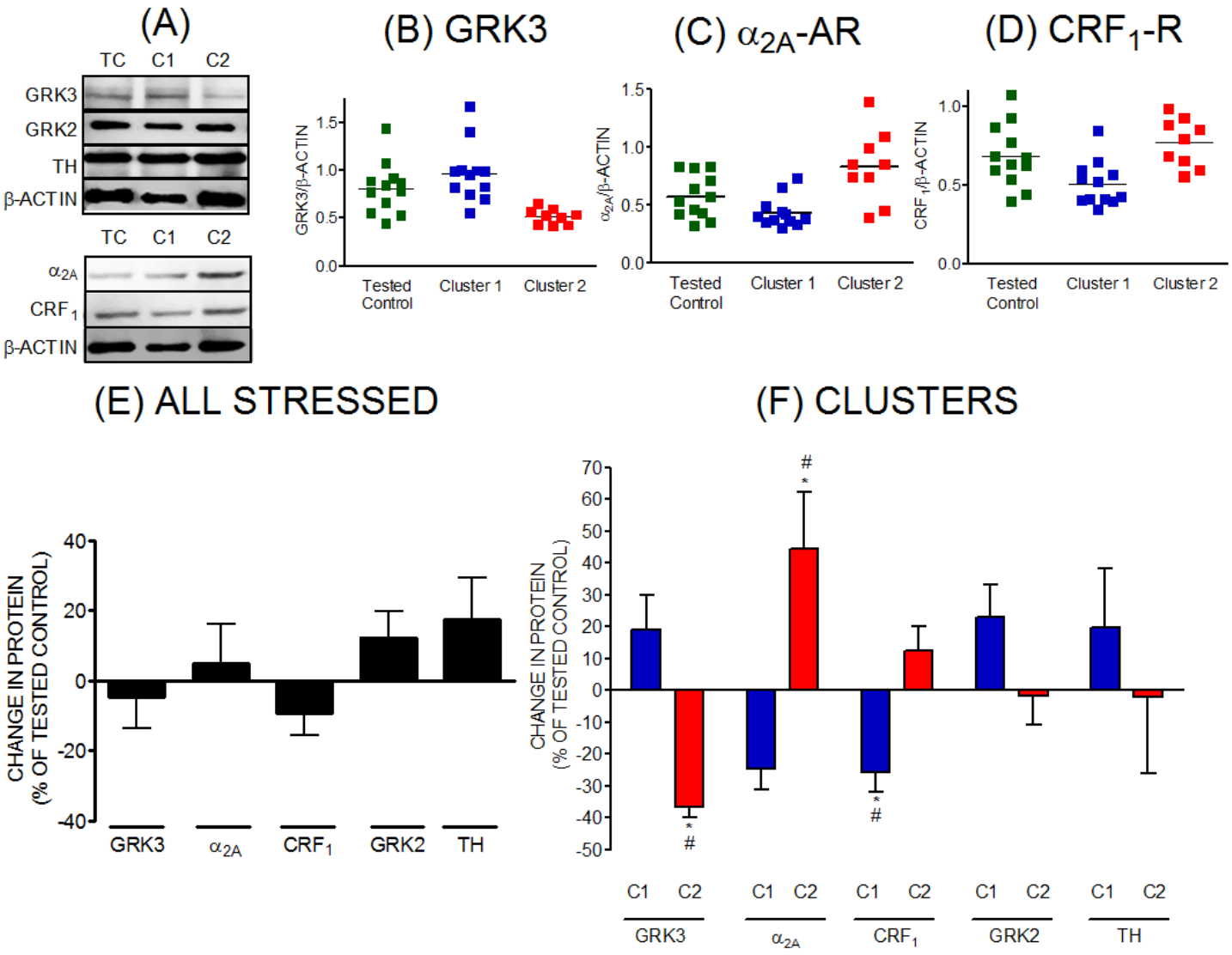

(A)
TC C1 C2
GRK3
GRK2
TH
β-ACTIN
TC C1 C2
α2A
CRF1
β-ACTIN
(B) GRK3
GRK3/β-ACTIN
Tested Control
Cluster 1
Cluster 2
(C) α2A-AR
α2A/β-ACTIN
Tested Control
Cluster 1
Cluster 2
(D) CRF1-R
CRF1/β-ACTIN
Tested Control
Cluster 1
Cluster 2
(E) ALL STRESSED
CHANGE IN PROTEIN (% OF TESTED CONTROL)
GRK3
α2A
CRF1
GRK2
TH
(F) CLUSTERS
CHANGE IN PROTEIN (% OF TESTED CONTROL)
C1 C2
GRK3
α2A
CRF1
GRK2
TH

Figure 13: **Changes in GRK3, α_{2A}-AR, and CRF_1 levels identify two clusters in LC 24h after single stress**

Rats were randomly separated into two groups. One group, the TC, was restrained but did not receive single bout of stress. The other group received single bout of (unpredictable, inescapable 100 tail shocks/ 1.2-2.0mA increased by 0.2mA/20shocks). Twenty four hour after single stress (no escape testing), rats were euthanized and brains were removed. LC was isolated from brains of TC, LH and NH rats and levels of GRK3, GRK2, TH , α_{2A}-AR and CRF_1-R were measured using western blot technique. Mean immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R, GRK2, TH /Loading control) ratios were calculated followed by change in protein for stressed rats and identified clusters C1, C2 alone are calculated in comparison to tested control.

Panel (A) Representative western blots for GRK3, GRK2, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (B, C, D)** Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios after cluster formations in comparison to tested control. **Panel (E)** Change in protein for all stressed animals (n=21) together expressed as % of TC (n=12). **Panel (F)** Change in protein for clusters identified from stressed animals expressed as % of TC. Significant change in protein levels ($p \leq 0.05$) *- (C2 vs.TC), and # - (C1 vs.C2) n=9-12.

Taken together, these data support the idea that single stress produces two populations of rats, one with significantly reduced levels of GRK3 in LC and the other with unchanged levels of GRK3 in LC. These populations are present without escape behavior testing in the shuttle box.

4.2.2 Segregation into two groups by cluster analysis: Amygdala 24h post- stress

Similar to LC, cluster analysis in amygdala based on GRK3, α_{2A}-AR, and CRF_1-R levels, revealed the existence of two significantly different clusters at 24h after single stress. In amygdala, when all stressed rats were compared to TC, there was a reduction of GRK3 (-12.4%) and a significant decrease of α_{2A}-AR (-30.5%; $p \leq 0.05$) and CRF_1-R (-18.8%) levels compared to TC **(Fig 14E)**. When the data was subjected to K-means cluster analysis using GRK3, α_{2A}-AR and CRF_1-R levels collectively as variables, two significantly different clusters were observed **(Fig 14B, C, D)**. The total number of stressed animals (21) was broken down into 2 clusters following cluster analysis: Cluster 1 (C1) having n=10 (48%) and cluster 2 (C2) having n=11 (52%). The cluster symmetry of amygdala with LC 24h-post stress data was 71.4% **(Fig 15)**. This uniformity suggests that cluster analysis identified similar clusters in both the LC and amygdala on the basis of GRK3, α_{2A}-AR and CRF_1-R levels.

After cluster analysis, the data for GRK3, α_{2A}-AR, and CRF_1-R was grouped according to the identified clusters (C1, C2 significantly different from

each other for each GRK3, α_{2A}-AR, and CRF_1-R levels) and compared to the TC group for each variable. Cluster C1 showed no changes in GRK3 levels with corresponding decrease in α_{2A}-AR (-46.8%; $p \leq 0.05$) and CRF_1-R (-49.2%; $p \leq 0.05$) levels when compared to TC **(Fig 14F)**. Cluster C2 show decrease in GRK3 levels (-22.1%; $p \leq 0.05$) accompanied by no significant change in α_{2A}-AR and CRF_1-R levels when compared to TC **(Fig 14F)**. The GRK3, α_{2A}-AR and CRF_1-R levels within the two clusters C1 and C2 were significantly different from each other. GRK2 and TH levels in amygdala were not significantly different between the clusters (C1 and C2) identified by the biochemical data.

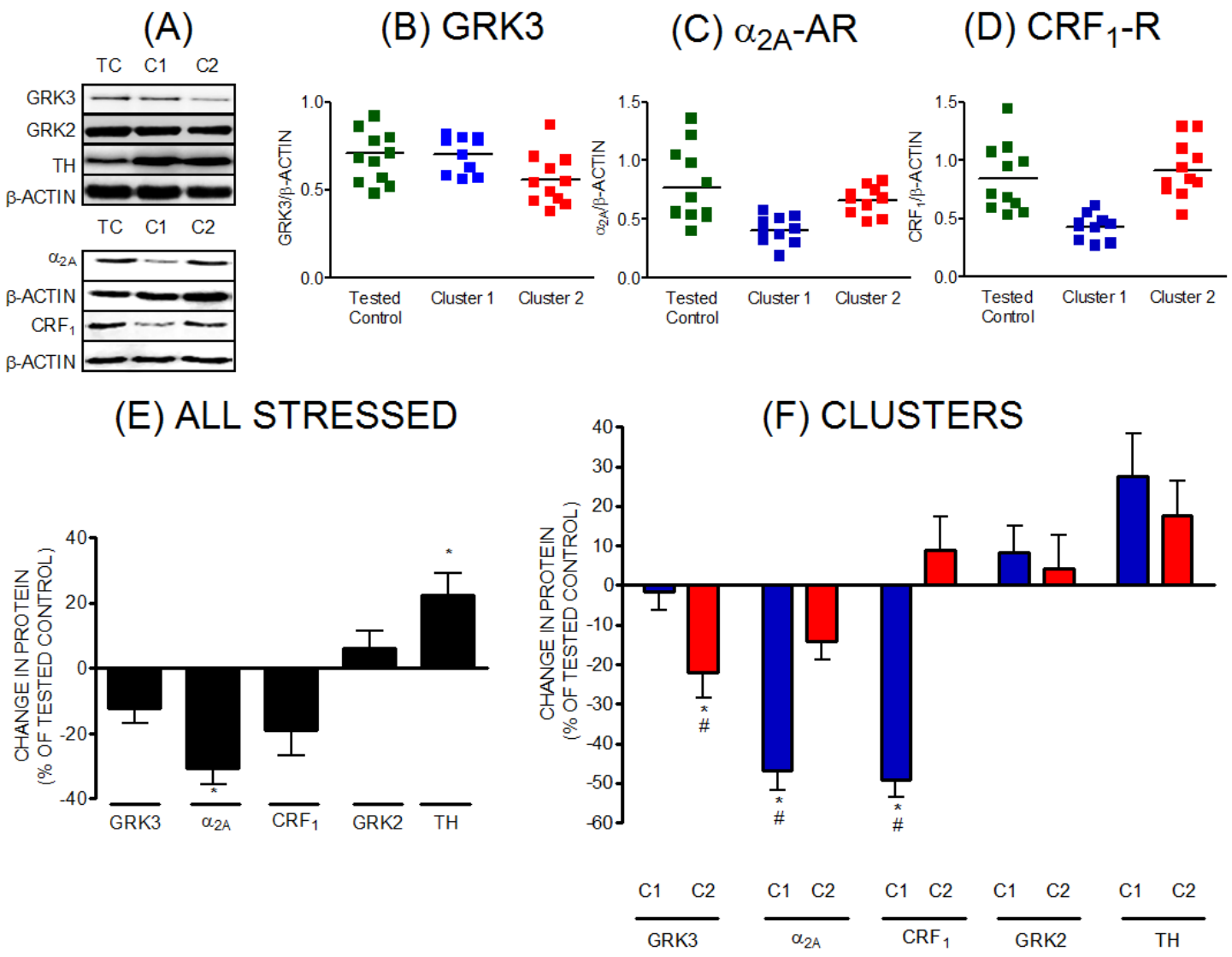

(A)
TC C1 C2
GRK3
GRK2
TH
β-ACTIN
TC C1 C2
α2A
β-ACTIN
CRF1
β-ACTIN
(B) GRK3
GRK3/β-ACTIN
1.0
0.5
0.0
Tested Control
Cluster 1
Cluster 2
(C) α2A-AR
α2A/β-ACTIN
1.5
1.0
0.5
0.0
Tested Control
Cluster 1
Cluster 2
(D) CRF1-R
CRF1/β-ACTIN
1.5
1.0
0.5
0.0
Tested Control
Cluster 1
Cluster 2
(E) ALL STRESSED
CHANGE IN PROTEIN (% OF TESTED CONTROL)
40
20
0
-20
-40
*
*
GRK3
α2A
CRF1
GRK2
TH
(F) CLUSTERS
CHANGE IN PROTEIN (% OF TESTED CONTROL)
40
30
20
10
0
-10
-20
-30
-40
-50
-60
* #
* #
* #
C1 C2
GRK3
C1 C2
α2A
C1 C2
CRF1
C1 C2
GRK2
C1 C2
TH

Figure 14: <u>Changes in GRK3, α_{2A}-AR, and CRF_1 levels identify two clusters in amygdala 24h after single stress</u>

Tested control (TC) and stressed rats, 24h post-stress (no escape testing), were euthanized and their brains were removed. Amygdala was isolated from brains of TC, stressed rats and levels of GRK3, GRK2, TH , α_{2A}-AR and CRF_1-R were measured using western blot technique **(For details refer to Fig 13 legend)**

.

Panel (A) Representative western blots for GRK3, GRK2, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (B, C, D)** Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios after cluster formations in comparison to tested control. **Panel (E)** Change in protein for all stressed animals (n=21) together expressed as % of TC (n=12). **Panel (F)** Change in protein for clusters identified from stressed animals expressed as % of TC. Significant change in protein levels ($p \leq 0.05$) *- (all stressed), C2 vs.TC), and # - (C1 vs.C2) n=10-12.

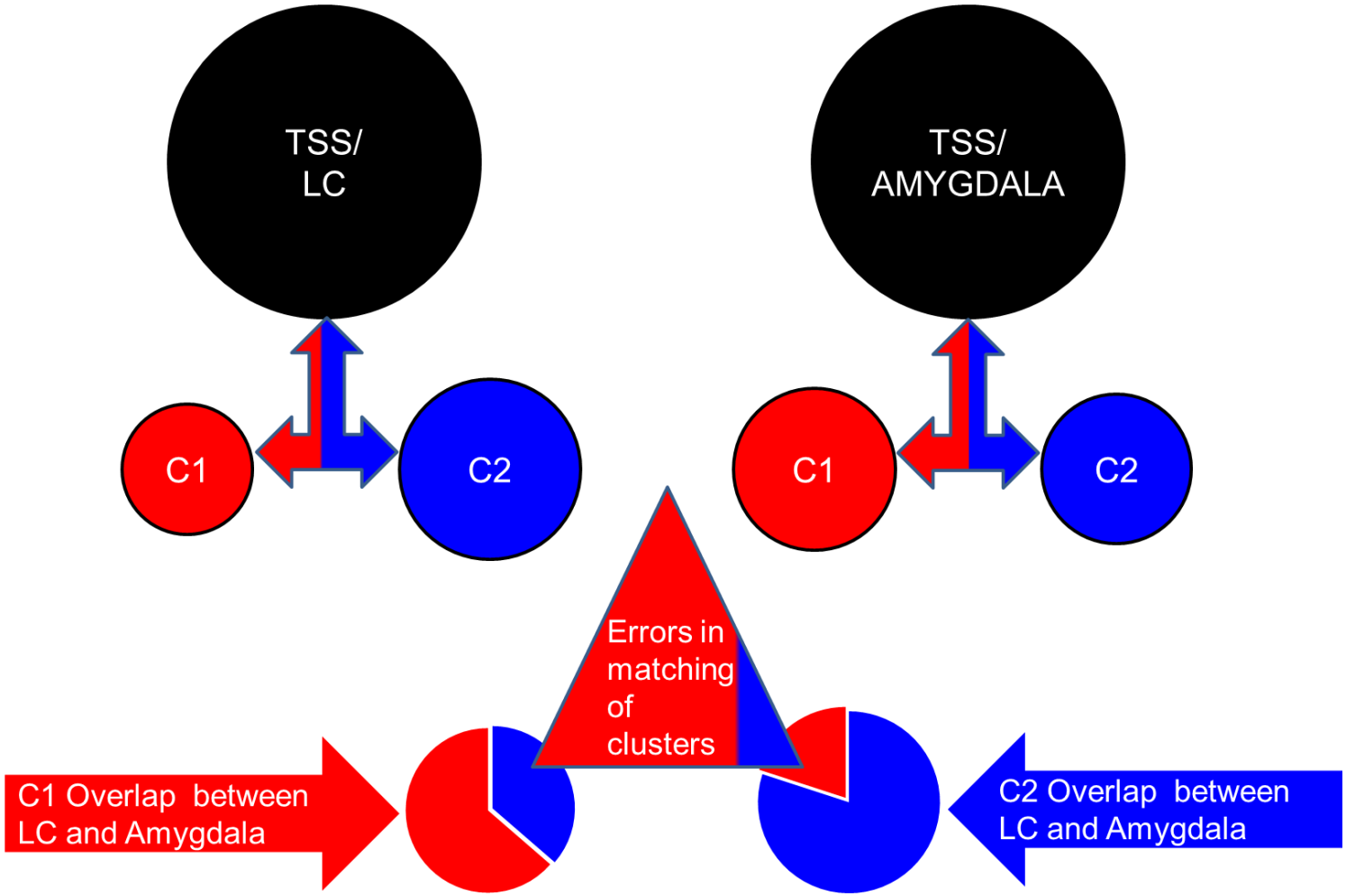

TSS/
LC
TSS/
AMYGDALA
C1
C2
C1
C2
Errors in
matching
of
clusters
C1 Overlap between
LC and Amygdala
C2 Overlap between
LC and Amygdala

Figure 15: Cluster Symmetry between locus coeruleus and amygdala 24h after single stress
Cluster symmetry estimated by % overlap between locus coeruleus and amygdala for C1 and C2 clusters was 71.4%. Error in matching of C1 cluster was 19.5% and error in matching of C2 cluster was 9.5%.

4.2.3 Non-segregation into two groups by cluster analysis: Cortex 24h post-stress

Although all stressed rats exhibited biochemical differences in protein levels in the cortex, cluster analysis of biochemical data failed to identify two different clusters suggesting that biochemical changes in cortex are stress dependent instead of behaviorally dependent. When all stressed rats were compared to TC, there was a reduction of GRK3 (-31.4%; $p \leq 0.05$) and a decrease in α_{2A}-AR (-20.5%) and CRF_1-R levels (-23.0%) **(Fig 16E)**. There was no change in GRK2 levels, but TH levels were reduced (-26.9%; $p \leq 0.05$) in stressed rats compared to TC **(Fig 16E)**.

In order to see that the effect of stress in cortex was similar within the stress group, rats were grouped on the basis of LC clusters, (C1, C2). Both clusters show reduction of GRK3 (-29.4%, -34.1%; $p \leq 0.05$) and decrease in α_{2A}-AR (-21.5%, -36.7%) and CRF_1-R (-31.9%, -28.4%) levels when compared to TC **(Fig 16F)**. When the data of stressed animals were segregated into two groups using LC clusters, there were no significant differences in GRK2 levels but TH levels show similar level of reduction (-27.8% C1, -26.0% C2). Therefore clustering the cortex data using clusters C1and C2 based on LC data show similar changes in both clusters **(Fig 16B, C, D)**.This confirms that the effects of stress in cortex cannot be clustered into two populations. The 24h post-stress

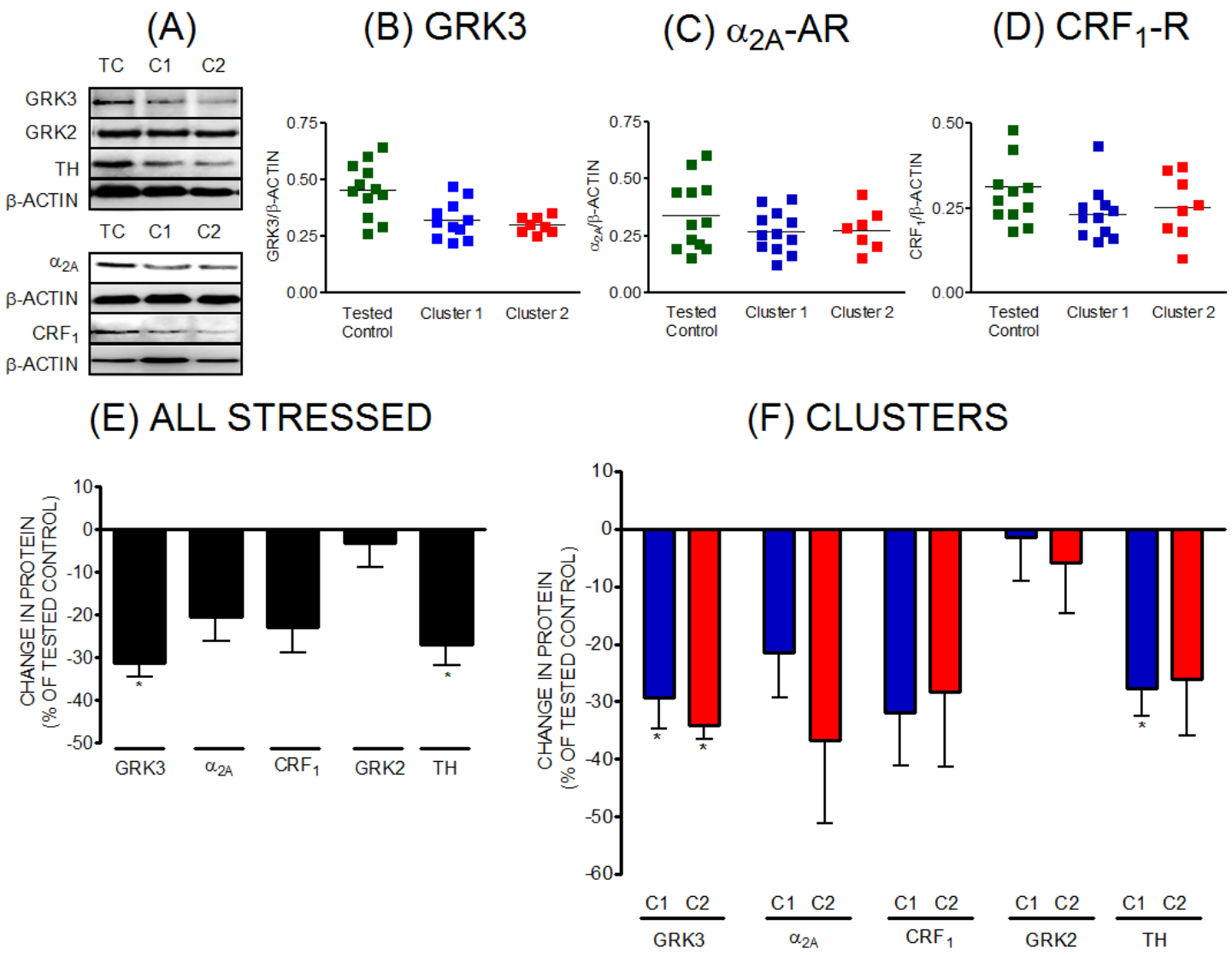

(A)
TC C1 C2
GRK3
GRK2
TH
β-ACTIN
TC C1 C2
α2A
β-ACTIN
CRF1
β-ACTIN
(B) GRK3
GRK3/β-ACTIN
0.75
0.50
0.25
0.00
Tested Control
Cluster 1
Cluster 2
(C) α2A-AR
α2A/β-ACTIN
0.75
0.50
0.25
0.00
Tested Control
Cluster 1
Cluster 2
(D) CRF1-R
CRF1/β-ACTIN
0.50
0.25
0.00
Tested Control
Cluster 1
Cluster 2
(E) ALL STRESSED
CHANGE IN PROTEIN (% OF TESTED CONTROL)
10
0
-10
-20
-30
-40
-50
*
*
GRK3
α2A
CRF1
GRK2
TH
(F) CLUSTERS
CHANGE IN PROTEIN (% OF TESTED CONTROL)
10
0
-10
-20
-30
-40
-50
-60
*
*
*
C1 C2
GRK3
C1 C2
α2A
C1 C2
CRF1
C1 C2
GRK2
C1 C2
TH

Figure 16: <u>Changes in GRK3, α_{2A}-AR, and CRF_1 levels do not identify two clusters in cortex 24h after single stress</u>

Tested control (TC) and stressed rats, 24h post-stress (no escape testing), were euthanized and their brains were removed. Cortex was isolated from brains of TC, stressed rats and levels of GRK3, GRK2, TH , α_{2A}-AR and CRF_1-R were measured using western blot technique **(For details refer to Fig 13 legend)**

Panel (A) Representative western blots for GRK3, GRK2, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (B, C, D)** Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios after cluster formations in comparison to tested control. **Panel (E)** Change in protein for all stressed animals (n=21) together expressed as % of TC (n=12). **Panel (F)** K-means cluster analysis of cortex data using GRK3, α_{2A}-AR, and CRF_1-R levels as collective variables did not identify two clusters. Therefore clustering for cortex data of stressed rats were based on LC biochemical data and change in protein is then expressed as % of TC. Significant change in protein levels ($p \leq 0.05$) *- (all stressed), C1, C2 vs.TC), n=9-12.

cortex data is in agreement with 48h post-stress data suggesting similar stress-dependent effects, but not exhibiting any group bifurcation.

4.3 Single stress-induced changes in GRK3, α_{2A}-AR and CRF_1-R levels precede development of LH behavior

The 48h post-stress data suggest that exposure to single stress leads to the identification of two clusters within stressed groups. One cluster has significantly reduced levels of GRK3 in LC and amygdala and relatively higher α_{2A}-AR and CRF_1-R levels while the other has unchanged levels of GRK3 in LC and amygdala and relatively reduced α_{2A}-AR and CRF_1-R levels. These clusters are present at 24h post-stress prior to escape behavior testing suggesting that changes in GRK3, α_{2A}-AR and CRF_1-R levels are present when LH or NH behavior also is first identified. Thus the time course of biochemical changes is mechanistically important and an earlier post-stress time point could help identify relationships between biochemical and behavioral changes. In order to understand this, rats were stressed and at 1h post-stress cluster analysis was used to identify the potential existence of two clusters within stressed rats.

4.3.1 Segregation into two groups by cluster analysis: LC 1h post-stress

Cluster analysis in LC based on 1h post-stress GRK3, α_{2A}-AR and CRF_1-R levels revealed the existence of two significantly different clusters. In LC, when

all stressed rats were compared to TC, there was a reduction of GRK3 (-24.0%; p≤0.05) and a corresponding (though not significant) elevation of α_{2A}-AR and CRF_1-R levels **(Fig 17E)**. The effect of stress does not appear to be global on all stressed rats, because when the data was subjected to K-means cluster analysis using GRK3, α_{2A}-AR and CRF_1-R levels as variables, two significantly different clusters appeared **(Fig 17B, C, D)**. The total numbers of stressed rats were 18 which cluster analysis broke into two clusters: Cluster 1 (C1) having n=8 (45.5%) and cluster 2 (C2) having n=10 (55.5%). C1 shows no changes in GRK3, α_{2A}-AR and CRF_1-R levels as compared to TC. C2 shows reduction of GRK3 (-42.8%; p≤0.05) with corresponding increase in α_{2A}-AR (+43.4%; p≤0.05) and CRF_1-R (+33.3%; p≤0.05) levels when compared to TC **(Fig 17F)**. There were no significant changes in GRK2 and TH levels at 1h post-stress whether all stressed rats were compared to TC or C1or C2 cluster within stressed rats was compared to TC **(Fig 17E, F)**.

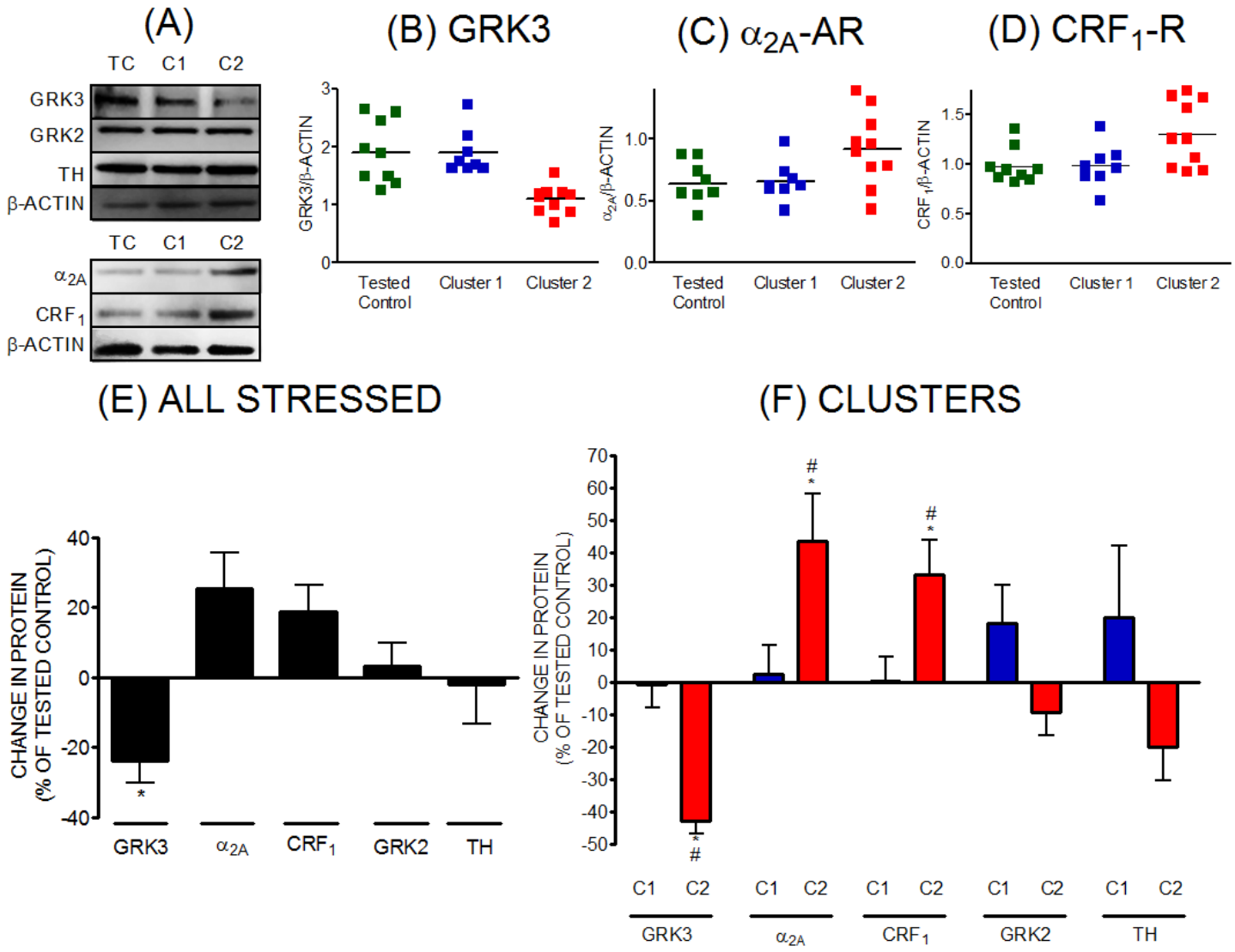

(A)
TC C1 C2
GRK3
GRK2
TH
β-ACTIN
TC C1 C2
α2A
CRF1
β-ACTIN
(B) GRK3
GRK3/β-ACTIN
Tested Control
Cluster 1
Cluster 2
(C) α2A-AR
α2A/β-ACTIN
Tested Control
Cluster 1
Cluster 2
(D) CRF1-R
CRF1/β-ACTIN
Tested Control
Cluster 1
Cluster 2
(E) ALL STRESSED
CHANGE IN PROTEIN (% OF TESTED CONTROL)
GRK3
α2A
CRF1
GRK2
TH
(F) CLUSTERS
CHANGE IN PROTEIN (% OF TESTED CONTROL)
C1 C2
GRK3
α2A
CRF1
GRK2
TH

Figure 17: <u>Changes in GRK3, α_{2A}-AR, and CRF_1 levels identify two clusters in LC 1h after single stress</u>

Rats were randomly separated into two groups. One group, the TC, was restrained but did not receive single bout of stress. The other group received single bout of (unpredictable, inescapable 100 tail shocks/ 1.2-2.0mA increased by 0.2mA/20shocks). One hour after single stress (no escape testing), rats were euthanized and brains were removed. LC was isolated from brains of TC, LH and NH rats and levels of GRK3, GRK2, TH , α_{2A}-AR and CRF_1-R were measured using western blot technique. Mean immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R, GRK2, TH /Loading control) ratios were calculated followed by change in protein for stressed rats and identified clusters C1, C2 alone are calculated in comparison to tested control.

Panel (A) Representative western blots for GRK3, GRK2, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (B, C, D)** Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios after cluster formations in comparison to tested control. **Panel (E)** Change in protein for all stressed animals (n=18) together expressed as % of TC (n=9).**Panel (F)** Change in protein for clusters identified from stressed animals expressed as % of TC. Significant change in protein levels ($p \leq 0.05$) *- (all stressed), C2 vs.TC), and # - (C1 vs.C2) n=8-10.

4.3.2 Non-segregation into two groups by cluster analysis: Amygdala 1h post-stress

In contrast to LC, cluster analysis of amygdala biochemical data failed to segregate the data into two different clusters. In amygdala, when all stressed rats were compared to TC, there was a reduction of GRK3 (-28.6%; $p \leq 0.05$) and a corresponding increase of α_{2A}-AR (+34.6 %; $p \leq 0.05$) and CRF_1-R (+54.8 %; $p \leq 0.05$) levels **(Fig 18E)**.The effect of stress appears global on all stressed rats, because when the data was subjected to K-means cluster analysis using GRK3, α_{2A}-AR and CRF_1-R levels collectively as variables, no significantly different clusters were identified **(Fig 18B, C, D)**. The stressed rat data from amygdala was grouped on the basis of clusters identified in LC. C1 and C2 both show reduction of GRK3 (-30.0%, -27.6%; $p \leq 0.05$) with corresponding increases in α_{2A}-AR (+35.6%, +33.7%- not significant) and CRF_1-R (+58.3%, +51.7%; $p \leq 0.05$) levels when compared to TC **(Fig 18F)**. The similarity of changes in amygdala data when clustered using the LC data suggests that the effects of stress at the level of amygdala were similar within the stressed group at 1h post-stress. There were no significant differences in GRK2 levels in amygdala. There was an increase in TH levels (+41.3%; $p \leq 0.05$) in stressed rats 1h post-stress and in both the clusters identified on the basis of LC (+38.7% C1, +43.3% C2) **(Fig 18E, F)**.

4.3.3 Non-segregation into two groups by cluster analysis: Cortex 1h post-stress

Similar to 24 and 48h post-stress data, the cortex of all stressed rats show changes in GRK3, α_{2A}-AR and CRF_1-R levels at 1h-post stress, but cluster analysis of stressed rat's biochemical data show that there are not two significantly different clusters. This again suggests that biochemical changes in cortex are stress dependent. In cortex, when all stressed rats were compared to TC, there was a reduction of GRK3 (-21.9%; $p \leq 0.05$) and a corresponding (though not significant) increase of α_{2A}-AR (+31.2%) and CRF_1-R (+31.9%) levels **(Fig 19E)**. There was a decrease in GRK2 (18%; $p \leq 0.05$) level and no change in TH level as compared to TC **(Fig 19E)**. To confirm that the effect of stress is global on all stressed rats, grouping of cortex biochemical data was done on the basis of LC clusters, and both clusters (C1, C2) show reduction of GRK3 (-11.3%, -30.3%) with corresponding increase in α_{2A}-AR (+16.8%, +42.6%) and CRF_1-R (+17.4%, +43.4%) levels when compared to TC **(Fig 19F)**. C2 show greater reduction in GRK3 and greater increase in α_{2A}-AR and CRF_1-R levels significantly different from TC, but both clusters C1and C2 were not statistically different from each other. When the data of stressed animals were segregated into two groups using LC clusters, there was no significant difference in GRK2 and TH level (**Fig 19F)**. The data at 1h post-stress in cortex does not show similar changes in both groups identified on the basis of LC clusters, which

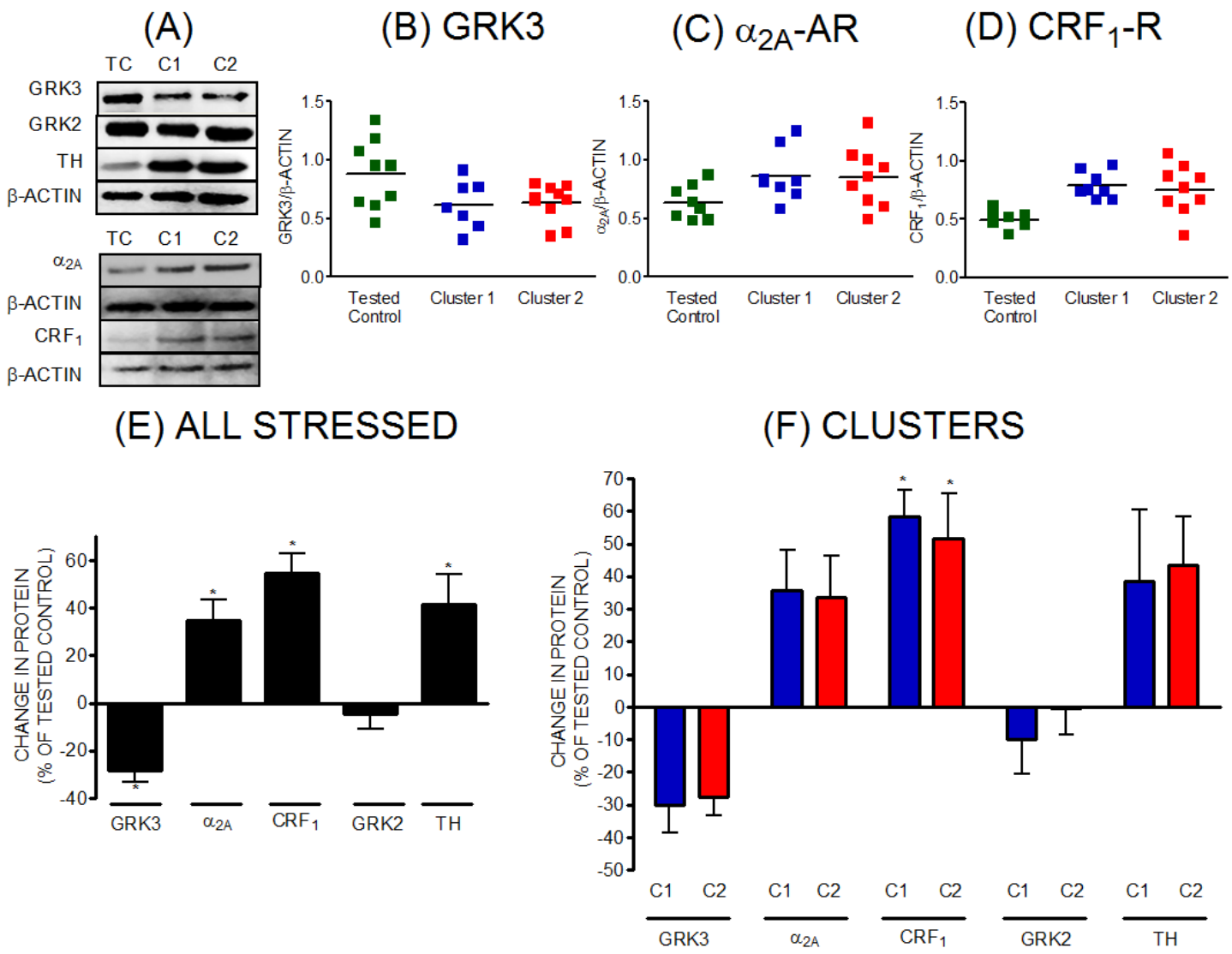

(A)
TC C1 C2
GRK3
GRK2
TH
β-ACTIN
TC C1 C2
α2A
β-ACTIN
CRF1
β-ACTIN
(B) GRK3
GRK3/β-ACTIN
1.5
1.0
0.5
0.0
Tested Control
Cluster 1
Cluster 2
(C) α2A-AR
α2A/β-ACTIN
1.5
1.0
0.5
0.0
Tested Control
Cluster 1
Cluster 2
(D) CRF1-R
CRF1/β-ACTIN
1.5
1.0
0.5
0.0
Tested Control
Cluster 1
Cluster 2
(E) ALL STRESSED
CHANGE IN PROTEIN (% OF TESTED CONTROL)
60
40
20
0
-20
-40
GRK3
α2A
CRF1
GRK2
TH
(F) CLUSTERS
CHANGE IN PROTEIN (% OF TESTED CONTROL)
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
C1 C2 C1 C2 C1 C2 C1 C2 C1 C2
GRK3
α2A
CRF1
GRK2
TH

Figure 18: Changes in GRK3, α_{2A}-AR, and CRF_1 levels do not identify two clusters in amygdala 1h after single stress

Tested control (TC) and stressed rats, 1h post-stress (no escape testing), were euthanized and their brains were removed. Amygdala was isolated from brains of TC, stressed rats and levels of GRK3, GRK2, TH , α_{2A}-AR and CRF_1-R were measured using western blot technique **(For details refer to Fig 17 legend)**

Panel (A) Representative western blots for GRK3, GRK2, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (B, C, D)** Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios after cluster formations in comparison to tested control. **Panel (E)** Change in protein for all stressed animals (n=18) together expressed as % of TC (n=9).**Panel (F)** K-means cluster analysis of cortex data using GRK3, α_{2A}-AR, and CRF_1-R levels as collective variables did not identify two clusters. Therefore clustering for amygdala data of stressed rats were based on LC biochemical data and change in protein is then expressed as % of TC. Significant change in protein levels ($p \leq 0.05$) *- (all stressed), C1, C2 vs.TC), n=8-10.

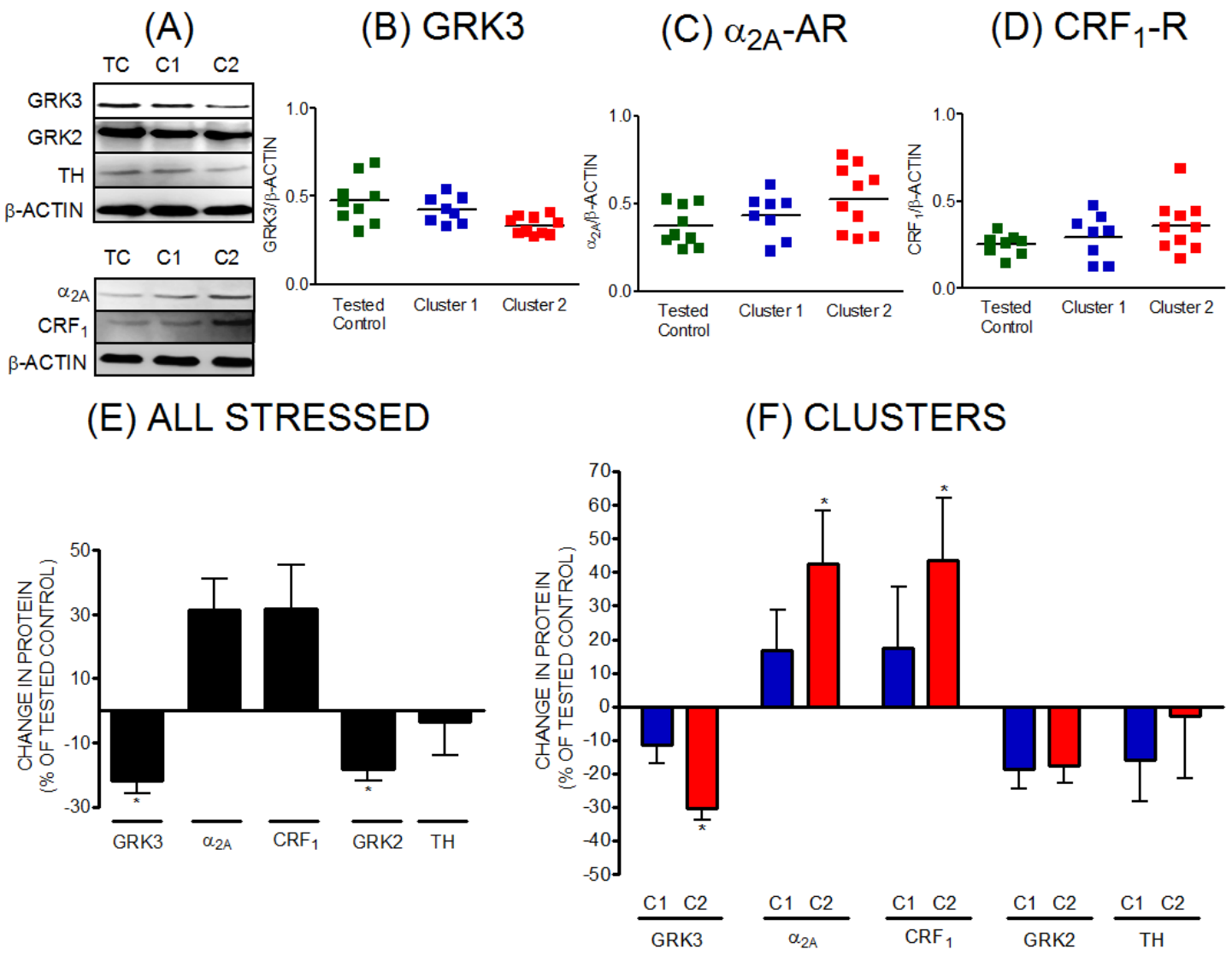

(A)
TC C1 C2
GRK3
GRK2
TH
β-ACTIN
TC C1 C2
α2A
CRF1
β-ACTIN
(B) GRK3
GRK3/β-ACTIN
1.0
0.5
0.0
Tested Control
Cluster 1
Cluster 2
(C) α2A-AR
α2A/β-ACTIN
1.0
0.5
0.0
Tested Control
Cluster 1
Cluster 2
(D) CRF1-R
CRF1/β-ACTIN
1.0
0.5
0.0
Tested Control
Cluster 1
Cluster 2
(E) ALL STRESSED
CHANGE IN PROTEIN (% OF TESTED CONTROL)
50
30
10
-10
-30
*
*
GRK3
α2A
CRF1
GRK2
TH
(F) CLUSTERS
CHANGE IN PROTEIN (% OF TESTED CONTROL)
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
*
*
C1 C2
GRK3
C1 C2
α2A
C1 C2
CRF1
C1 C2
GRK2
C1 C2
TH

Figure 19: <u>Changes in GRK3, α_{2A}-AR, and CRF_1 levels do not identify two clusters in cortex 1h after single stress</u>

Tested control (TC) and stressed rats, 1h post-stress (no escape testing), were euthanized and their brains were removed. Cortex was isolated from brains of TC, stressed rats and levels of GRK3, GRK2, TH , α_{2A}-AR and CRF_1-R were measured using western blot technique **(For details refer to Fig 17 legend)**

Panel (A) Representative western blots for GRK3, GRK2, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (B, C, D)** Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios after cluster formations in comparison to tested control. **Panel (E)** Change in protein for all stressed animals (n=18) together expressed as % of TC (n=9).**Panel (F)** K-means cluster analysis of cortex data using GRK3, α_{2A}-AR, and CRF_1-R levels as collective variables did not identify two clusters. Therefore clustering for cortex data of stressed rats were based on LC biochemical data and change in protein is then expressed as % of TC. Significant change in protein levels ($p \leq 0.05$) *- (all stressed), C1, C2 vs.TC), n=8-10.

is not in agreement with 48h post stress data wherein changes were similar in LH and NH rats.

4.4 Disappearance of learned helpless behavior-5 days post-stress (single stress)

Twenty-four hours after single stress (1 day post-stress) or after restraint for TC, mean escape latencies and escape failures were determined as indices of escape behavior. K-means cluster analysis of escape behavior data identified two significantly different populations among stressed rats. One exhibited escape latencies and escape failures similar to TC (NH behavior) and other exhibited larger escape latencies and escape failures than TC and NH (LH behavior) **(Fig 20A, B)**. A second escape testing was done 5 days post-stress. Stressed rats exhibiting LH behavior 1 day post-stress showed similar escape behavior as NH and TC rats 5 days post-stress. The data suggest that LH behavior after single stress is transient and reversible by 5 days post-stress **(Fig 20A, B)**.

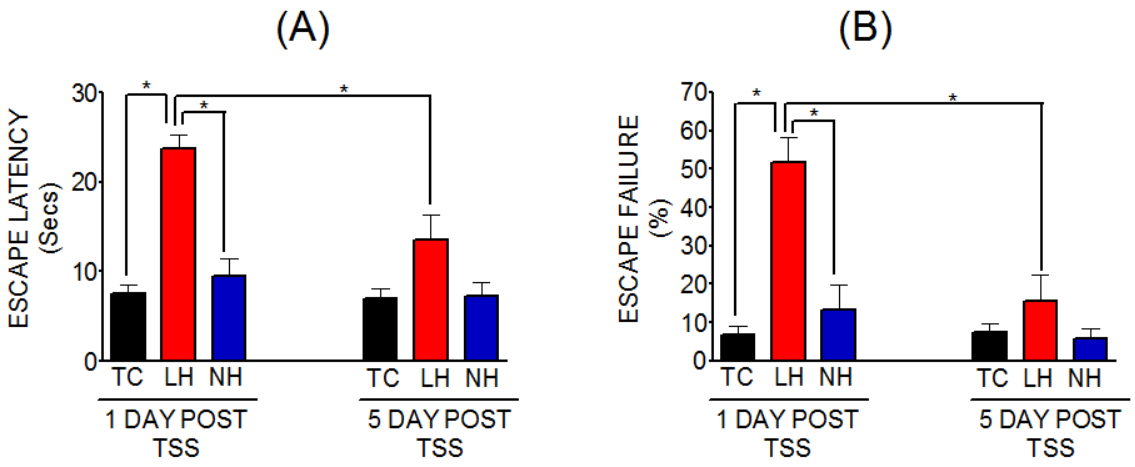


Figure 20: <u>Escape behavior data of single stressed rats showing disappearance of LH behavior 5 day post-stress (120h post-stress)</u>
Rats were randomly separated into two groups. One group, the TC, was restrained but did not receive single bout of stress. The other group received single bout of stress (unpredictable, inescapable 100 tail shocks/ 1.2-2.0mA increased by 0.2mA/20shocks). Twenty four hour after single stress or after restraint for TC escape latencies and escape failures were determined by escape testing. Escape latencies and escape failures data of stressed rats subjected to K-means cluster analysis, identified the presence of two populations within stressed rats (LH-higher, NH-similar escape latencies/escape failures to TC). Five days post-stress (120h post-stress), second escape testing and similar cluster analysis was done.

Panel (A, B) Escape latencies, escape failures of LH, NH rats identified by cluster analysis and presence of two populations were confirmed by comparison with TC rats. One day post-stress, rats (n=12) showed a bimodal distribution of 5 LH rats (greater escape latencies/failures than TC rats) and 7 NH rats (escape latencies/failures similar to TC rats). Five day post-stress (120h post-stress) behavior data no longer show bimodal distribution of LH and NH rats. Data is expressed as Mean ± SEM, and * - mean escape latency/failure significantly different (LH vs. NH), (LH vs.TC) (LH 1day vs. 5 days post-stress ($p \leq 0.05$) (n=5-12).

4.5 Changes in GRK3, α_{2A}-AR and CRF_1-R levels- 6 days post-stress (single stress)

4.5.1 LC: 6 days post-stress

Disappearance of LH behavior resulted in changes in GRK3, α_{2A}-AR and CRF_1-R levels that can no longer be discriminated between LH and NH rats. GRK3 levels were increased in LH and NH rats (+27.0%, +33.8%; $p \leq 0.05$) respectively compared to TC **(Fig 21E)**. Levels of α_{2A}-AR were down-regulated in LH rats (-34.5%; $p \leq 0.05$) compared to TC, but not in NH rats (-15.5%) **(Fig 21E)**. However, CRF_1-R levels were down regulated in both LH and NH rats (-25.9%, -24.6%; $p \leq 0.05$) respectively **(Fig 21E)**. Thus at 6 days post-stress when there is no behavioral discrimination between LH and NH rats, differences in levels of GRK3, α_{2A}-AR, CRF_1-R in the LC disappear. In contrast to GRK3, GRK2 levels in both LH and NH were unchanged compared to TC **(Fig 21E)**. TH levels were increased in the LC of LH rats (+30.7%), whereas they were decreased in NH rats (-19.1%) when compared to TC rats.

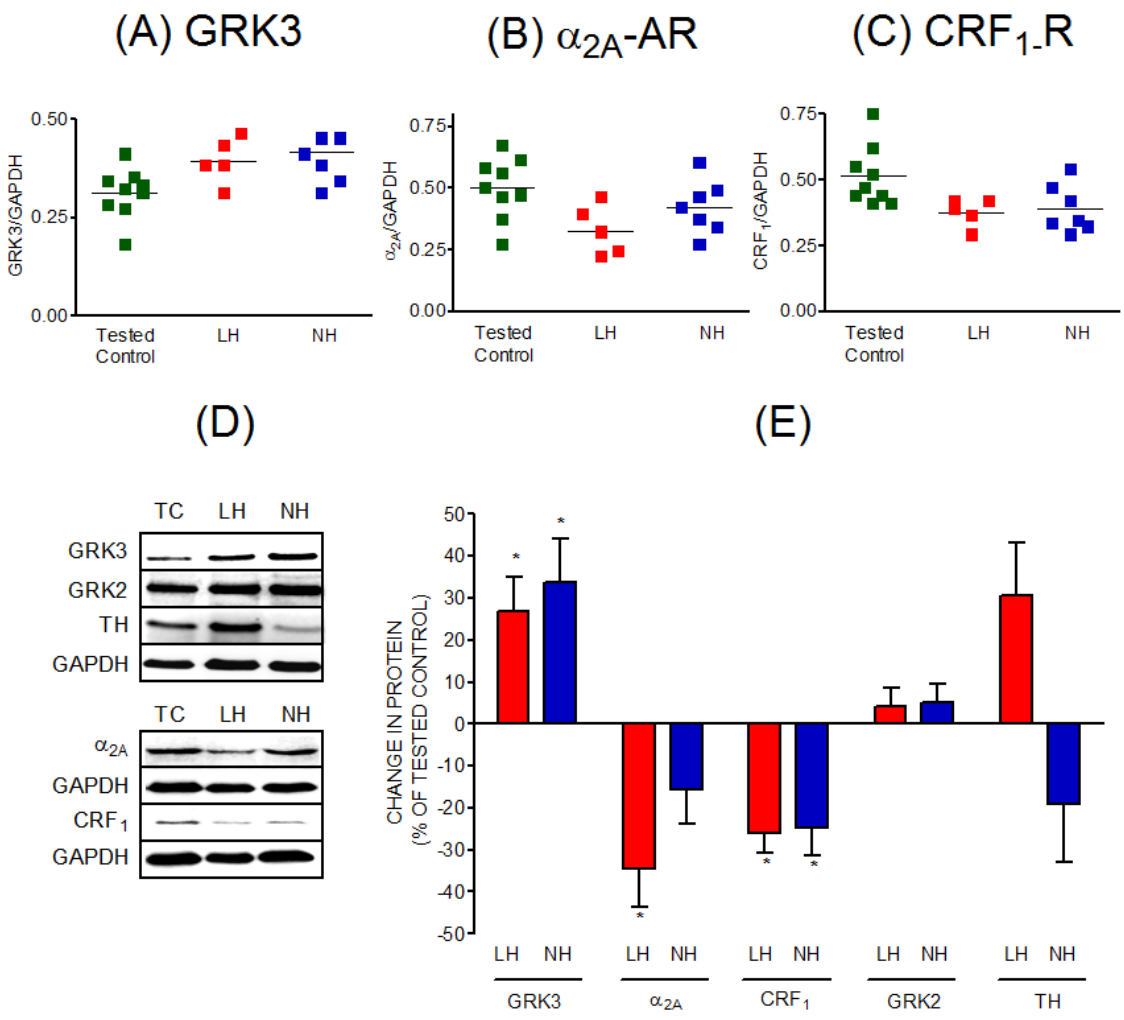

(A) GRK3
(B) α2A-AR
(C) CRF1-R
GRK3/GAPDH
α2A/GAPDH
CRF1/GAPDH
0.50
0.25
0.00
0.75
Tested Control
LH
NH
(D)
(E)
TC
LH
NH
GRK3
GRK2
TH
GAPDH
α2A
CRF1
CHANGE IN PROTEIN (% OF TESTED CONTROL)
50
40
30
20
10
0
-10
-20
-30
-40
-50
*
GRK3
α2A
CRF1
GRK2
TH

Figure 21: Changes in GRK3, α_{2A}-AR, and CRF_1 levels in locus coeruleus 6 days post-stress (single stress)

Rats were randomly separated into two groups. One group, the TC, was restrained but did not receive single bout of stress. The other group received single bout of stress (unpredictable, inescapable 100 tail shocks/ 1.2-2.0mA increased by 0.2mA/20shocks). Twenty four hour and 6 day after stress , escape latencies and escape failures data of stressed rats was subjected to K-means cluster analysis, to identify the presence of two populations within stressed rats (LH-higher, NH-similar escape latencies/escape failures to TC). Six day post-stress (24h after second escape testing), rats were euthanized and brains were removed. LC was isolated from brains of TC, LH and NH rats and levels of GRK3, GRK2, TH , α_{2A}-AR and CRF_1-R were measured using western blot technique. Mean immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R, GRK2, TH /Loading control) ratios were calculated followed by change in protein for LH, NH rats alone are calculated in comparison to tested control.

Panel (A, B, C) Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios of LH, NH rats in comparison to tested control. **Panel (D)** Representative western blots for GRK3, GRK2, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (E) C**hange in protein for LH and NH rats expressed as % of tested control. Significant change in protein levels ($p \leq 0.05$) *- (LH, NH vs.TC), n=5-9.

4.5.2 Amygdala: 6 days post-stress

Similar to LC, disappearance of LH behavior also resulted in non-behavioral specific changes in GRK3, α_{2A}-AR, and CRF_1-R levels in amygdala. GRK3 levels were increased in NH rats (+39.0%; p≤0.05) and LH rats (+23.8%) respectively, compared to TC **(Fig 22E)**. Levels of α_{2A}-AR were down-regulated in NH rats (-32.3%; p≤0.05) compared to TC, but not in in LH rats (-10.6%) **(Fig 22E)**. Levels of CRF_1-R were also down-regulated in NH rats (-29.3%; p≤0.05) compared to TC, but not in LH rats **(**-12.3%) **(Fig 22E)**. GRK2 and TH levels in both LH and NH were unchanged compared to TC **(Fig 22E)**. Although NH rats exhibited moderately larger changes than LH rats compared to TC, no significant differences in GRK3, α_{2A}-AR, and CRF_1-R were detected between NH and LH rats.

4.5.3 Cortex: 6 days post- stress

GRK3, α_{2A}-AR, CRF_1-R, GRK2, and TH levels in both LH and NH were not different from each other suggesting that there is no biochemical distinction between LH and NH rats **(Fig 23E)**. These protein levels were also unchanged compared to TC, thus whatever stress dependent effects have occurred post-stress 48h are no longer present 144h post-stress.

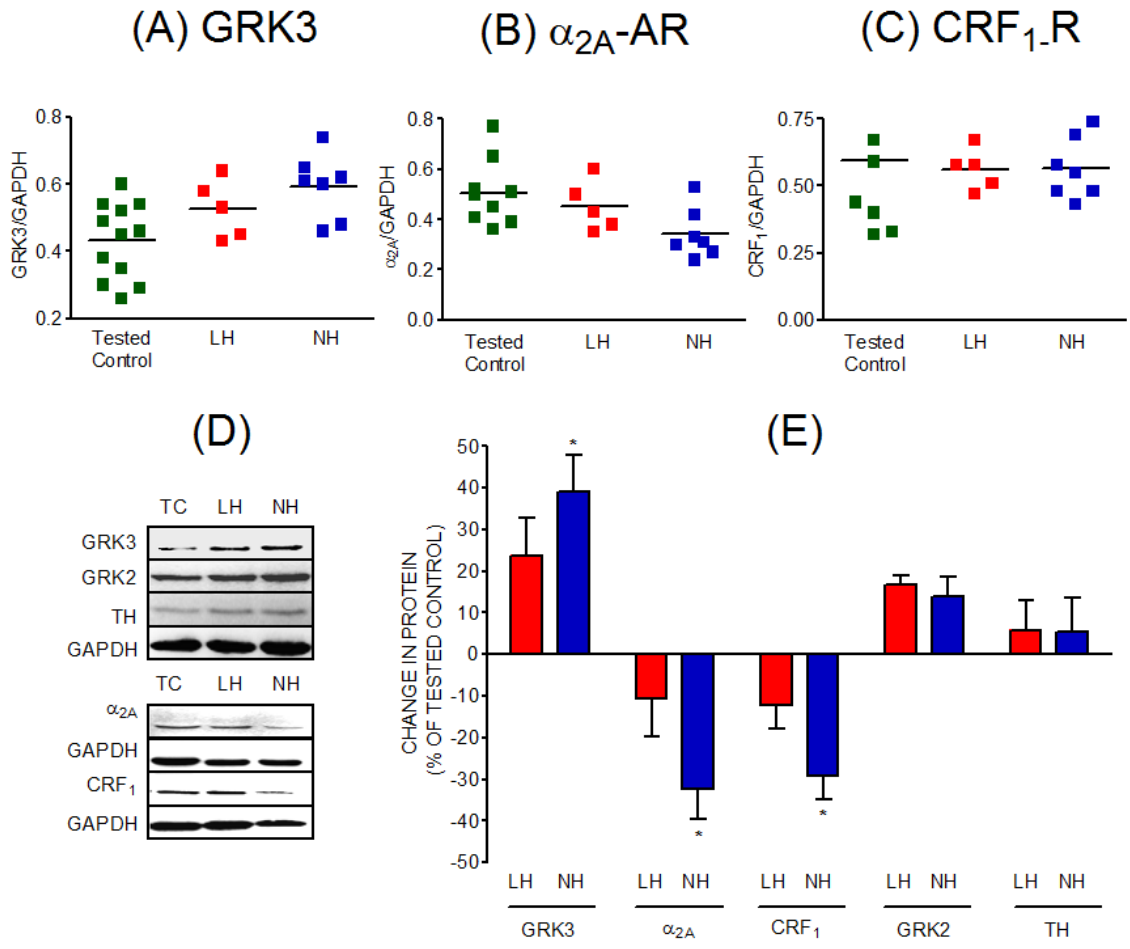

(A) GRK3
(B) α2A-AR
(C) CRF1-R
GRK3/GAPDH
α2A/GAPDH
CRF1/GAPDH
Tested Control
LH
NH
(D)
TC LH NH
GRK3
GRK2
TH
GAPDH
α2A
CRF1
(E)
CHANGE IN PROTEIN (% OF TESTED CONTROL)
GRK3
α2A
CRF1
GRK2
TH

Figure 22: Changes in GRK3, α_{2A}-AR, and CRF_1 levels in amygdala 6 days post-stress (single stress)

Tested control (TC) and stressed rats, 6 days post-stress (24h after second escape testing), were euthanized and brains were removed. Amygdala was isolated from brains of TC, LH and NH rats and levels of GRK3, GRK2, TH , α_{2A}-AR and CRF_1-R were measured using western blot technique **(For details refer to Fig 21 legend)**.

Panel (A, B, C) Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios of LH, NH rats in comparison to tested control. **Panel (D)** Representative western blots for GRK3, GRK2, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (E)** Change in protein for LH and NH rats expressed as % of tested control. Significant change in protein levels ($p \leq 0.05$) *- (LH, NH vs.TC), n=5-12.

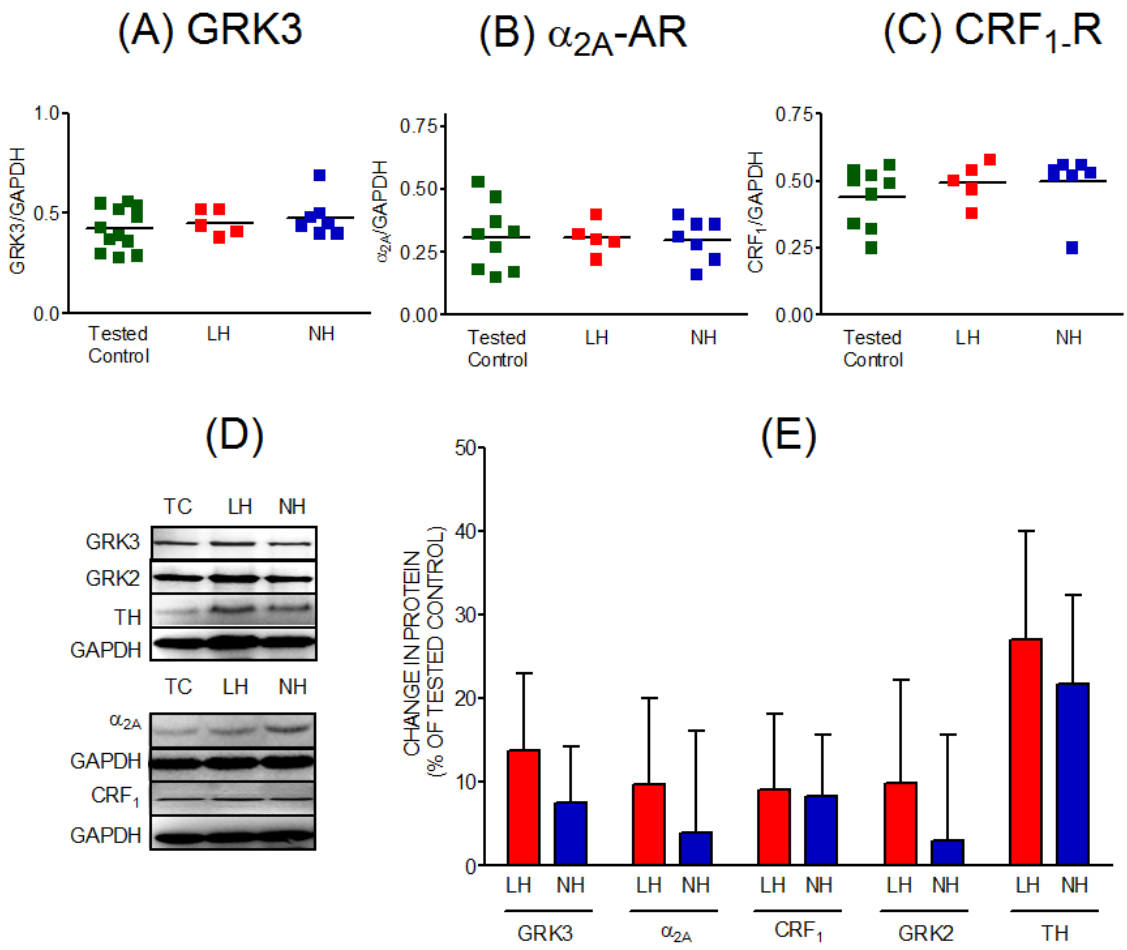

(A) GRK3
(B) α2A-AR
(C) CRF1-R
GRK3/GAPDH
α2A/GAPDH
CRF1/GAPDH
1.0
0.5
0.0
0.75
0.50
0.25
0.00
Tested Control
LH
NH
(D)
TC
LH
NH
GRK3
GRK2
TH
GAPDH
α2A
CRF1
(E)
CHANGE IN PROTEIN (% OF TESTED CONTROL)
50
40
30
20
10
0
GRK3
α2A
CRF1
GRK2
TH

Figure 23: No change in GRK3, α_{2A}-AR, and CRF_1 levels in cortex 6 days post-stress (single stress)

Tested control (TC) and stressed rats, 6 days post-stress (24h after second escape testing), were euthanized and brains were removed. Cortex was isolated from brains of TC, LH and NH rats and levels of GRK3, GRK2, TH , α_{2A}-AR and CRF_1-R were measured using western blot technique **(For details refer to Fig 21 legend)**.

Panel (A) Representative western blots for GRK3, GRK2, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (B, C, D)** Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios of LH, NH rats in comparison to tested control. **Panel (E)** Change in protein for LH and NH rats expressed as % of tested control n=5-12.

4.6 Repeated stress and prolonged learned helpless behavior

The single period of stress induces learned helpless (LH) behavior for 3 days only. Analysis of escape behavior data for single stress showed LH behavior 24h post-stress in 50% of rats. When the same LH rats were tested for escape deficits 5 days after the single stress, the animals no longer showed LH behavior. LH rats behaved similar to tested controls. Taking the time course of reversal of LH behavior into consideration tail shock stress was administered at 3 day intervals and a model of repeated stress-induced prolonged LH behavior was developed. LH behavior persisted at least 14 days. Since these studies involved repeated exposure of stress, rats were restrained 3 times in the tested control group. In order to control for this possible confounding stress, an additional control group, naïve control (NC), was included where rats were neither restrained nor shocked but were otherwise handled the same way as TC and repeated stressed groups of rats.

4.6.1 Repeated stress-induced 14 day prolonged LH behavior

Rats exposed to three bouts of stress on days 1, 4, 7 exhibited significantly greater FR2 trial escape latencies and escape failures in comparison to TC or NC rats (**Fig 24A, B)** on day 8 and day 22 post-stress. However when day 8 and day 22 post-stress FR2 trial escape behavior data of all stressed rats were subjected to K-means cluster analysis, the data showed a

bimodal distribution, indicating the presence of two different populations within the repeated stressed group of rats. One population had similar mean escape latencies and escape failures compared to controls (NC or TC); in other words they exhibited NH behavior. The other population had significantly greater mean escape latencies and escape failures compared to NH, NC or TC groups. Therefore these rats exhibited LH behavior (given a chance these rats cannot escape from an escapable situation). Furthermore, the LH behavior persisted at least until day 22 post-stress when the second escape test was done (**Fig 24C, D)**. Similarly escape behaviors of NH, NC and TC rats were similar on days 8 and 22 post-stress and significantly different from the LH population. The FR1 trials on days 8 and 22 post-stress with the easier escape task showed no differences across NC, TC, LH, and NH groups suggesting that locomotor deficits among LH rats can be ruled out as the cause of the greater escape deficits of LH rats in the FR2 trials.

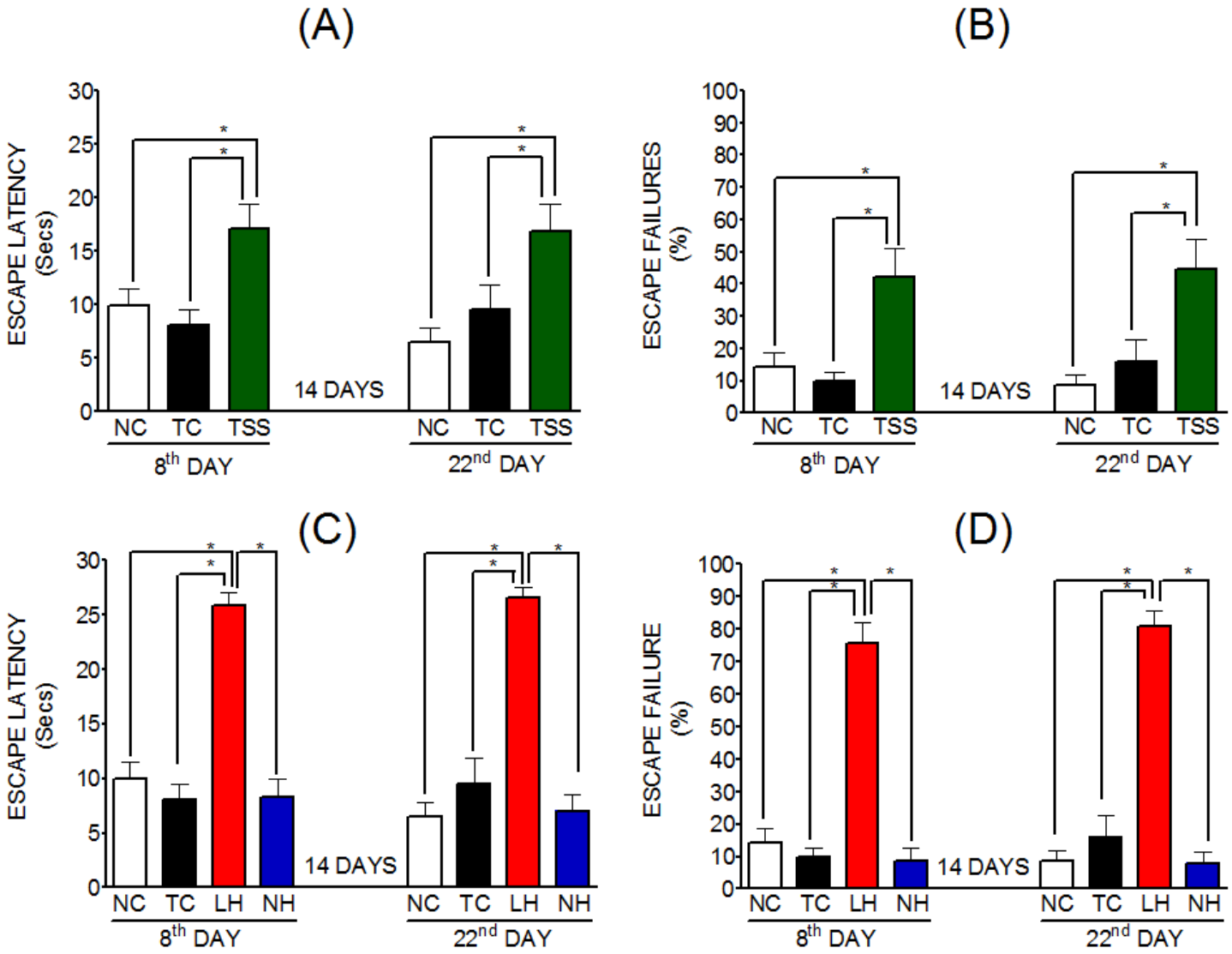

(A)
ESCAPE LATENCY (Secs)
30
25
20
15
10
5
0
NC TC TSS
14 DAYS
NC TC TSS
8th DAY
22nd DAY
(B)
ESCAPE FAILURES (%)
100
90
80
70
60
50
40
30
20
10
0
NC TC TSS
14 DAYS
NC TC TSS
8th DAY
22nd DAY
(C)
ESCAPE LATENCY (Secs)
30
25
20
15
10
5
0
NC TC LH NH
14 DAYS
NC TC LH NH
8th DAY
22nd DAY
(D)
ESCAPE FAILURE (%)
100
90
80
70
60
50
40
30
20
10
0
NC TC LH NH
14 DAYS
NC TC LH NH
8th DAY
22nd DAY

Figure 24: Escape behavior data of repeated stress rats bifurcates into two distinct behaviors persisting for 14 days

Rats were randomly separated into three groups. First group, naïve control (NC), was restrained did not receive repeated stress. Second group, TC, was restrained on days 1, 4, 7 but did not receive repeated stress. Third group received repeated stress (unpredictable, inescapable 100 tail shocks/ 1.2-2.0mA increased by 0.2mA/20shocks) on days 1, 4, 7. On day 8 and 22 escape latencies and escape failures were determined by escape testing for rats in each group. Day 8 escape latencies and escape failures data of repeated stress rats was subjected to K-means cluster analysis, which identified the presence of two populations within repeatedly stressed rats (LH-higher, NH-similar escape latencies/escape failures to TC or NC). Day 22 post-stress, rats exhibiting LH behavior on day 8 post-stress showed a similar level of escape latencies and escape failures to those recorded earlier.

Panel (A) Comparison of day 8 and 22 post-stress, mean escape latency of NC, TC, and repeated tail shock stress (TSS) rats. **Panel (B)** Comparison of day 8 and 22 post-stress, mean escape failure of NC, TC, and repeated tail shock stress (TSS) rats . **Panel (C, D)** Escape latencies and escape failures of LH, NH rats identified by cluster analysis of day 8 and 22 post-stress data and presence of two populations were confirmed by comparison with NC or TC rats. Repeatedly stressed rats (n=18) showed a bimodal distribution of 9 LH rats (greater escape latencies/failure than NC or TC rats) and 9 LH rats (escape latencies/failure similar to NC or TC rats). Data is expressed as Mean ± SEM, and * - mean escape latency and escape failure significantly different (LH vs. NH), (LH vs.TC), and (LH day 8 vs. day 22 post-stress) ($p \leq 0.05$) n=9-12.

4.6.2 Repeated stress-induced 14 day prolonged LH behavior: Assessment of behavior in other paradigms

In order to assess if repeated stress-induced sustained LH behavior is confounded by anxiety, locomotor deficits or decreased exploratory behavior, these parameter also were assessed. Moreover, a different inescapable stressful paradigm was utilized to determine whether LH and NH rats behave differently in this paradigm.

4.6.2.1 Open field test (OFT)

The OFT was conducted to examine the locomotor and exploratory activity of control (NC, TC), NH and LH rats. Day 15 post-stress, when LH and NH behavior among repeated stress rats was conserved, OFT showed that there was no significant difference between the locomotor activity (indicated by total, vertical stereo and ambulatory activity) of NC, TC, LH and NH rats **(Fig 25A)**. Similarly there was no significant difference between the exploratory activity (indicated by head rotations, rearing, and stereotypical bursts) of NC, TC, LH and NH rats **(Fig 25B)**. The OFT also can provide some measure of anxiety. In the OFT the total area can be divided into central and peripheral compartments. Less time spent and distance travelled within the central compartment as compared to the peripheral area and a reduced number of rat entries into the central compartment, all are indicative of anxious behavior. There were no significant

differences in these indices of anxiety shown between control rats (NC, TC) and LH /NH rats **(Fig 25C)**.

4.6.2.2 Elevated plus maze (EPM) test

The EPM test was applied to control (NC, TC) and NH/LH rats since it represents a more robust test for assessing behavioral substrates related to anxiety. The test utilizes the fear of rats towards open elevated areas and their preference to stay in closed spaces compared to open spaces as an index of anxiety behavior. More time spent in and more entries made into the closed area are indicative of anxious behavior. Control (NC, TC) and repeated stress (LH, NH) rats exhibited similar indices of anxiety behavior **(Fig 26A, B)**.

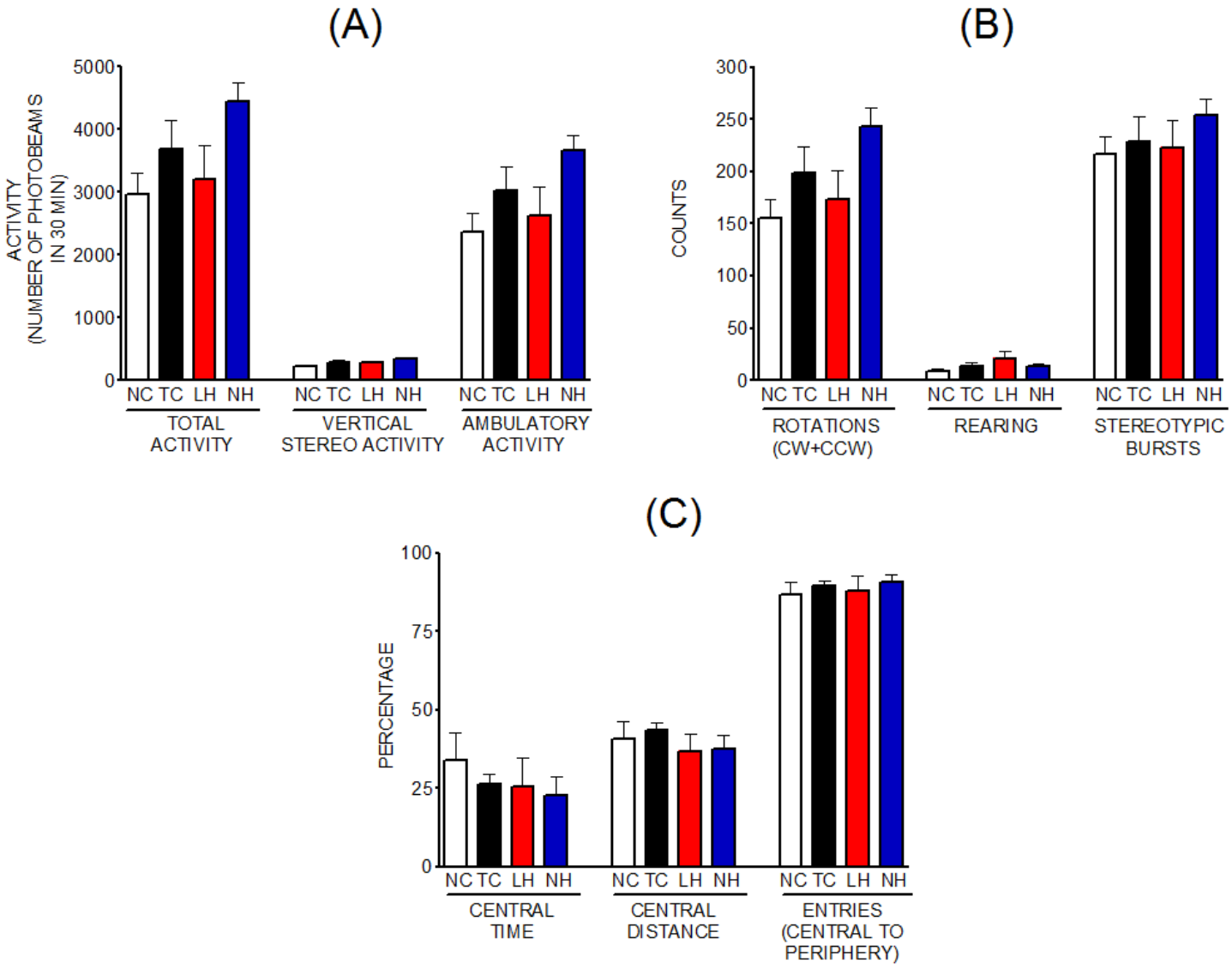

(A)
ACTIVITY
(NUMBER OF PHOTOBEAMS
IN 30 MIN)
5000
4000
3000
2000
1000
0
NC TC LH NH
TOTAL
ACTIVITY
NC TC LH NH
VERTICAL
STEREO ACTIVITY
NC TC LH NH
AMBULATORY
ACTIVITY
(B)
COUNTS
300
250
200
150
100
50
0
NC TC LH NH
ROTATIONS
(CW+CCW)
NC TC LH NH
REARING
NC TC LH NH
STEREOTYPIC
BURSTS
(C)
PERCENTAGE
100
75
50
25
0
NC TC LH NH
CENTRAL
TIME
NC TC LH NH
CENTRAL
DISTANCE
NC TC LH NH
ENTRIES
(CENTRAL TO
PERIPHERY)

Figure 25: <u>Open field test (OFT) indicates similar level of locomotor activity, exploratory activity and anxiety behavior in control (NC/TC) and repeatedly stressed (LH/NH) rats on day 15 post-stress</u>
OFT for measuring locomotor activity, exploratory activity and anxiety behavior was done on day 15 (8 days after last bout of tail shocks), in all groups of rats. First group, naïve control (NC), was restrained did not receive repeated stress. Second group, TC, was restrained on days 1, 4, 7 but did not receive repeated stress. Third group received repeated stress (unpredictable, inescapable 100 tail shocks/ 1.2-2.0mA increased by 0.2mA/20shocks) on days 1, 4, 7. On day 8 (24h after last bout of tail shocks) escape latencies and escape failures were determined by escape testing for rats in each group; the presence of two populations was confirmed by comparison with NC or TC rats.

Variables indicating locomotor activity **Panel (A)**,exploratory activity **Panel (B)**, and anxiety behavior **Panel (C)** of rats across control (TC, NC) and repeated stress (LH,NH) groups**.** Data is expressed as Mean ± SEM for panel A and B. Data for panel C is calculated as % time spent in, total distance travelled in, and total entries made in central compartment only compared to central and peripheral compartments together n=7-11.

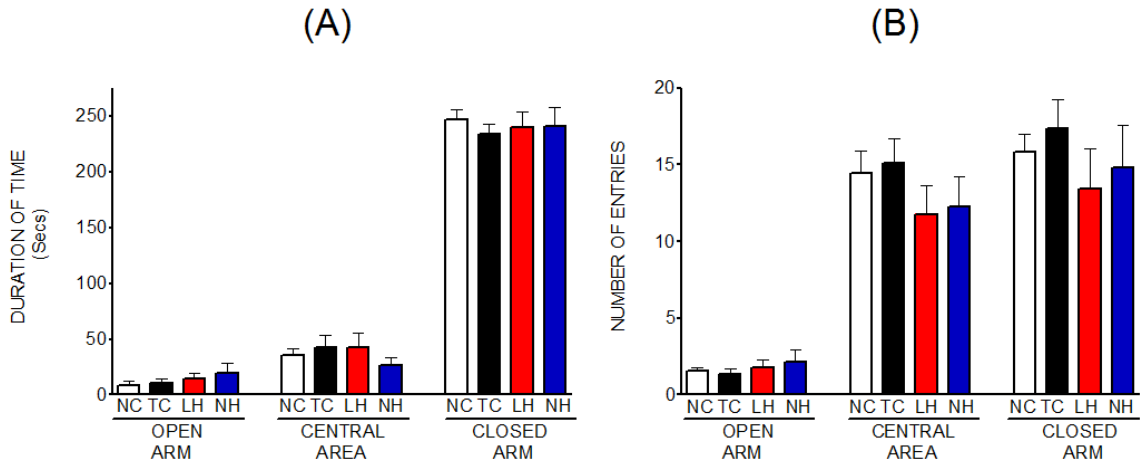


Figure 26: <u>Elevated plus maze (EPM) indicates similar level of anxiety behavior in control (NC/TC) and repeatedly stressed (LH/NH) rats as open field test on day 16 post-stress</u>

EPM test for measuring anxiety behavior was done on day 16 (9 days after last bout of TSS), in all groups of rats **(For details about groups and stress protocol refer to Fig 25 legend).**

Panel (A) Time spent and **Panel (B)** Number of entries made in designated areas of elevated plus maze by control (TC, NC) and repeated stress (LH,NH) rats. Data is expressed as Mean ± SEM n=7-11.

4.6.2.3 Modified forced swim stress test

In order to cross validate repeated stress-induced prolonged behaviors, a different stress paradigm, the modified forced swim stress (MFSS) was used to assess whether LH and NH rats behavior is sustained in a different paradigm. The test is based on an inescapable stress where a rat is put in cylinder filled with water for 15 minutes. The rat is observed for this time period and its behavior is assessed for 3 parametric evaluations: swimming, climbing (active behaviors-indicative of attempts to escape the situation) and immobility (passive behavior-indicative of despair). Analysis of the first five minutes of swim stress suggested that there were no differences in swimming and climbing behavior across the groups **(Fig 27A)**. Although LH rats show significantly higher immobility than TC rats, LH, NH and NC rats show similar level of immobility in first five minutes of testing **(Fig 27A)**. Analysis of 5-10, and 10-15 minutes of swim stress, showed a significant decrease in swimming and a significant increase in immobility of LH as compared to NC, TC and NH rats **(Fig 27B, C)**. Cumulative results of all 15 minutes together exhibited the same pattern of reduced swimming and increased immobility of LH rats compared to NC, TC and NH rats **(Fig 27D;** $p \leq 0.05$**)**. The behavioral data from the MFSS test suggest that the intensity and inescapable nature of stress leads to behavioral discrimination among repeatedly stressed rats, regardless of testing paradigm.

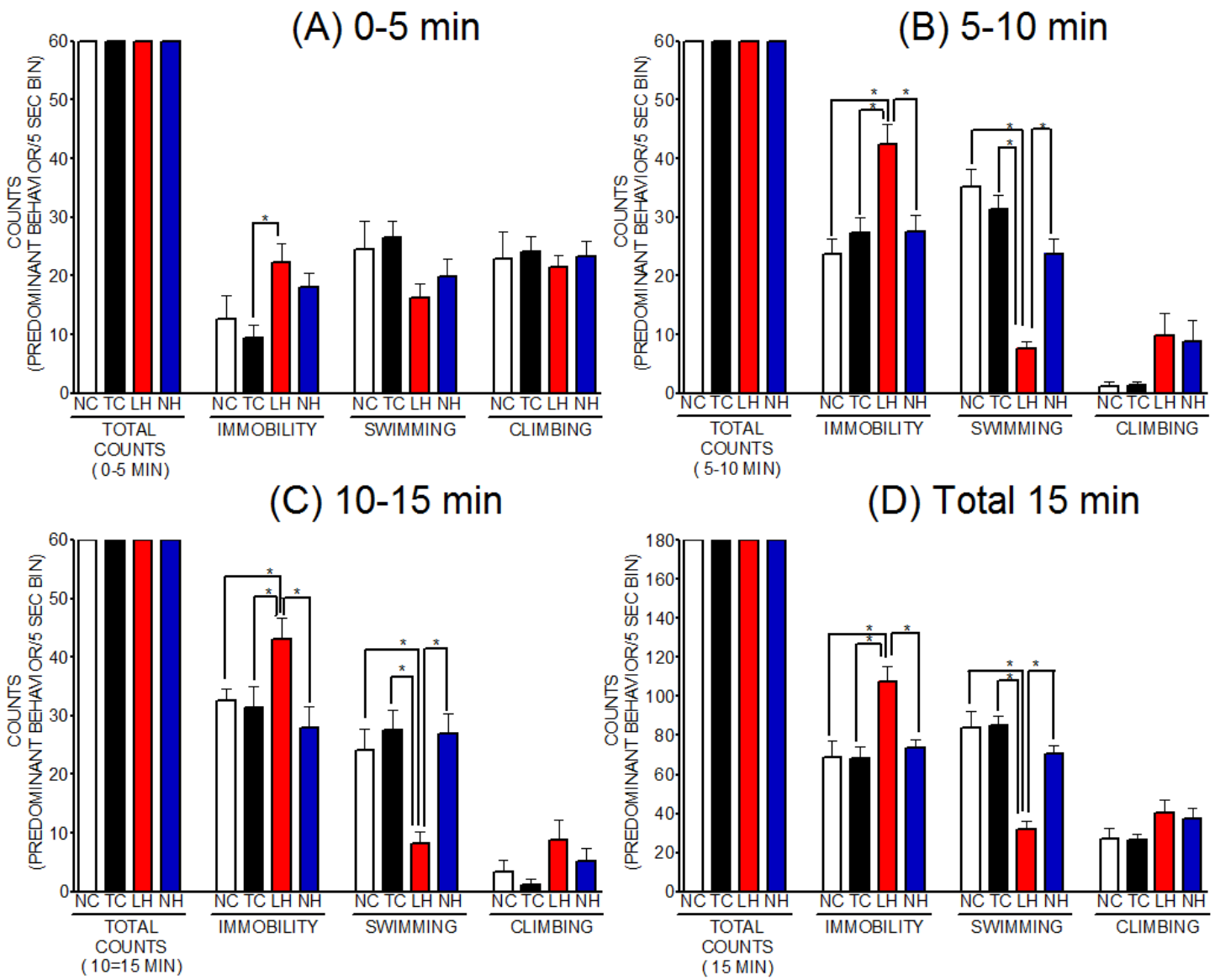

(A) 0-5 min
(B) 5-10 min
(C) 10-15 min
(D) Total 15 min
COUNTS
(PREDOMINANT BEHAVIOR/5 SEC BIN)
NC TC LH NH
TOTAL COUNTS (0-5 MIN)
IMMOBILITY
SWIMMING
CLIMBING
TOTAL COUNTS (5-10 MIN)
TOTAL COUNTS (10=15 MIN)
TOTAL COUNTS (15 MIN)

Figure 27: <u>Behavioral distinction of repeatedly stressed rats by modified forced swim stress (MFSS) test: Cross validation by another inescapable stress paradigm on day 17 post-stress</u>

MFSS test for measuring swimming, climbing (indicative of escape behavior), and immobility behavior (indicative of behavioral despair) was done on day 17 (9 days after last bout of TSS), in all groups of rats. . For behavioral scoring, the 15-min test was divided into 5-s bins, and scored for the predominant behavior for each bin: immobility, swimming, or climbing. The total number of scoring counts for each behavior for each rat was determined, and a mean count for each behavior is determined for each group. The comparisons were done for 0-5min, 5-10 min, 10-15 min and the total 15 min test period **(For details about groups and stress protocol refer to Fig 25 legend).**

Panel (A) 0-5 minutes , **Panel (B)** 5-10 minutes , **Panel (C)** 10-15 minutes , and **Panel (D)** Total 15 minutes break up of immobility, swimming, and climbing behavior of NC, TC, LH, and NH rats. Data is expressed as Mean ± SEM for behavioral counts. * - Immobility, swimming behavior significantly different (LH vs. NH), (LH vs.TC), and (LH vs.NC), $p \leq 0.05$ n=7-11.

4.6.3 Repeated stress-induced 14 day prolonged LH behavior: Predictive validity indicator with classical tricyclic antidepressant desipramine.

The single stress bout-induced LH model is used quite extensively for screening of clinically effective antidepressants. Most of the available antidepressants can prevent and/or reverse single stress bout-induced LH behavior. Fourteen days of LH behavior in rats approximately equals 1.5 years of clinical depression in humans. Therefore a drug that is more effective in cases of prolonged and severe depression (desipramine) was selected to attempt reversal of the sustained LH behavior in order to test its effectiveness in our repeated stress model.Rats exposed to three bouts of tail shock stress on days 1, 4, 7 exhibited significantly higher FR2 trial escape latencies and escape failures in comparison to NC or TC rats (**Fig 28A, B)** on day 8 post-stress as noted before. Desipramine treatment (5mg/kg, bid) from day 8-day 22 significantly reduced FR2 trial escape latencies and escape failures of repeatedly stress (3 bouts of TSS) rats. There were no significant changes in escape latencies and escape failures of repeatedly stress (3 bouts of TSS) rats as compared to NC, TC rats on day 22 post-stress. Escape latencies and escape failures on day 22 post-stress after desipramine treatment were significantly reduced in repeatedly stressed rats compared to day 8 post-stress **(Fig 28C,D)**.

K-Means cluster analysis of day 8 post-stress FR2 trial escape latencies and escape failures showed approximately 50% LH (n=5) and NH (n=7) rats

among a group of 12 repeatedly stressed rats (**Fig 28C, D)**. LH rats have significantly greater escape latencies and escape failures as compared to NC, TC, and NH rats. After treatment with desipramine differences in escape behaviors of LH, NH, TC and NC rats were abolished **(Fig 28C, D)**. Escape latencies and escape failures on day 22 post-stress after drug treatment were significantly reduced in LH rats as compared to day 8 post-stress **(Fig 28C,D)**. Desipramine treatment had no effect on day 22 post–stress escape latencies and escape failures of NC, TC, and NH rats compared to day 8 post-stress.

4.6.4 Changes in stress hormones in repeatedly stress-induced LH behavior and its reversal by desipramine treatment: Face validity indicator of model

Blood was collected by tail vein on day 8 post-stress (before first escape testing) and day 23 (24h after second escape testing). On day 8 post-stress norepinephrine **(Fig 29A),** epinephrine **(Fig 29B),** and corticotrophin releasing factor (CRF) levels **(Fig 29C)** were similar across the groups while corticosterone levels **(Fig 29D)** were significantly higher in LH rats. On day 23 post-stress epinephrine **(Fig 29B)**, CRF **(Fig 29C)**, and corticosterone levels **(Fig 29D)** were significantly higher in LH rats. Fourteen days of desipramine treatment (5mg/Kg, bid) from day 8 to 22 post-stress normalized epinephrine, CRF and corticosterone levels on day 23 **(Fig 29B, C, D)**.

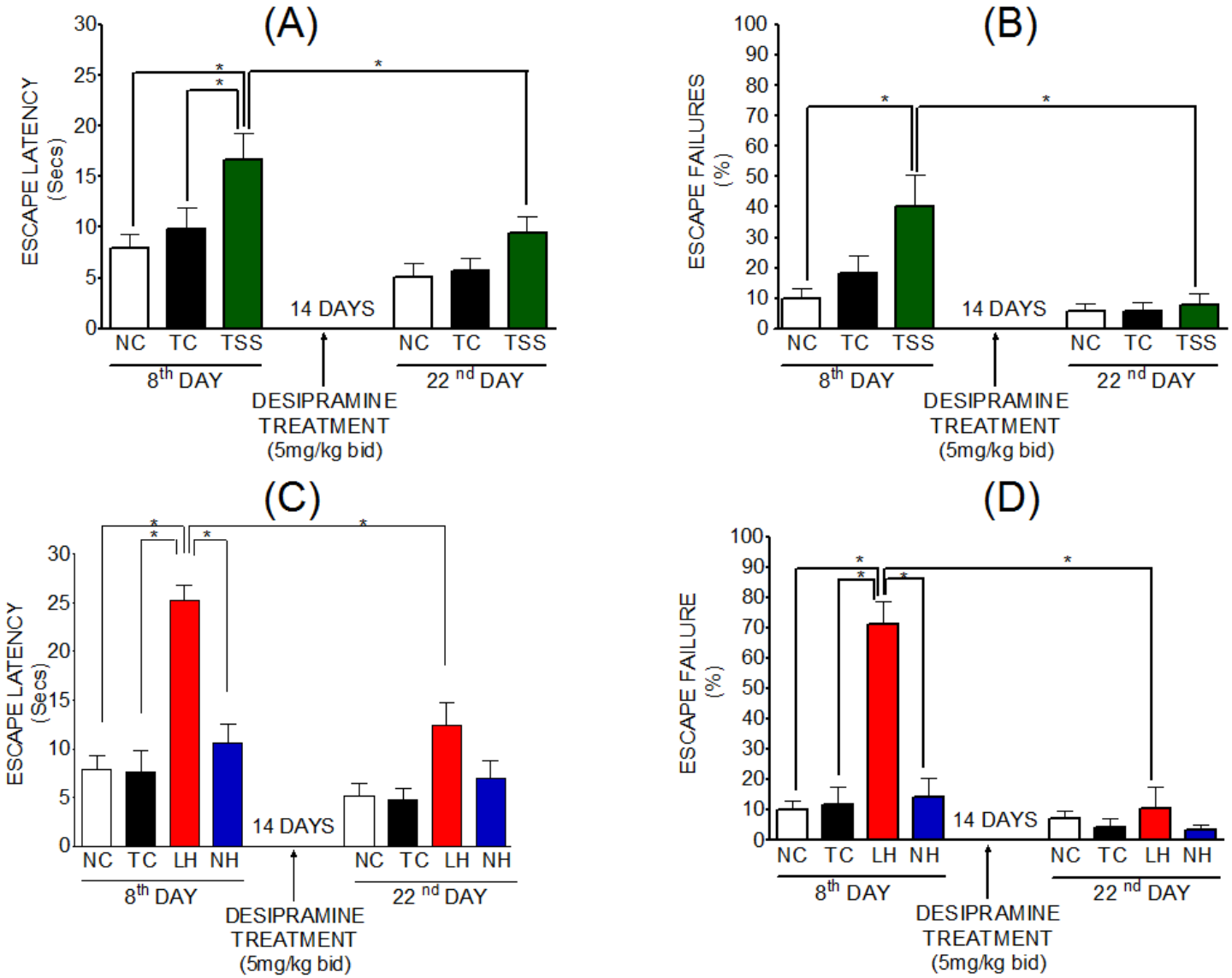

(A)
ESCAPE LATENCY (Secs)
30
25
20
15
10
5
0
*
*
*
NC TC TSS
14 DAYS
NC TC TSS
8th DAY
22 nd DAY
DESIPRAMINE TREATMENT (5mg/kg bid)
(B)
ESCAPE FAILURES (%)
100
90
80
70
60
50
40
30
20
10
0
*
*
NC TC TSS
14 DAYS
NC TC TSS
8th DAY
22 nd DAY
DESIPRAMINE TREATMENT (5mg/kg bid)
(C)
ESCAPE LATENCY (Secs)
30
25
20
15
10
5
0
*
*
*
*
NC TC LH NH
14 DAYS
NC TC LH NH
8th DAY
22 nd DAY
DESIPRAMINE TREATMENT (5mg/kg bid)
(D)
ESCAPE FAILURE (%)
100
90
80
70
60
50
40
30
20
10
0
*
*
*
*
NC TC LH NH
14 DAYS
NC TC LH NH
8th DAY
22 nd DAY
DESIPRAMINE TREATMENT (5mg/kg bid)

Figure 28: <u>Reversal of repeated stress-induced prolonged learned helplessness behavior by 14 days of desipramine treatment</u>

Rats were randomly separated into three groups. First group, naïve control (NC), was restrained did not receive repeated stress. Second group, TC, was restrained on days 1, 4, 7 but did not receive repeated stress. Third group received repeated stress (unpredictable, inescapable 100 tail shocks/ 1.2-2.0mA increased by 0.2mA/20shocks) on days 1, 4, 7. On day 8 escape latencies and escape failures were determined by escape testing for rats in each group. Day 8 escape latencies and escape failures data of repeated stress rats was subjected to K-means cluster analysis, which identified the presence of two populations within repeatedly stressed rats (LH-higher, NH-similar escape latencies/escape failures to TC or NC). Fourteen days of desipramine treatment (5mg/kg, bid i.p.) from day 8 (after first escape testing) -day 22 (before second escape testing) was given to controls (NC/TC) and repeatedly stress (LH/NH) rats

Panel (A) Comparison of day 8 (before desipramine treatment) and day 22 post-stress (after 14 days of desipramine treatment) mean escape latency of NC, TC, and repeated stress rats. **Panel (B)** Comparison of day 8 (before desipramine treatment) and day 22 post-stress (after 14 days of desipramine treatment) mean escape failure of NC, TC, and repeated stress rats. **Panel (C, D)** Escape latencies and escape failures of LH, NH rats identified by cluster analysis of day 8 post-stress data and presence of two populations were confirmed by comparison with NC or TC rats. Repeated stress (n=12) rats showed a bimodal distribution of 5 LH rats (greater escape latencies/failure than NC or TC rats) and 7 NH rats (escape latencies/failure similar to NC or TC rats). After 14 days of desipramine treatment repeated stress rats no longer show bimodal distribution demonstrated by similar escape behavior of LH rats compared to NH, NC, and TC rats. K-means cluster analysis of day 22 post-stress data revealed non-existence of two populations, therefore data of day 22 post-stress data is clustered on the basis of LH/NH population identified on day 8 post-stress (before desipramine treatment). Data is expressed as Mean ± SEM, and * - mean escape latency/failure significantly different (LH vs. NH), (LH vs.TC), (LH vs. NC), and {LH day 8 (before desipramine treatment) vs. day 22 (after drug treatment) post-stress) ($p \leq 0.05$) n=5-6.

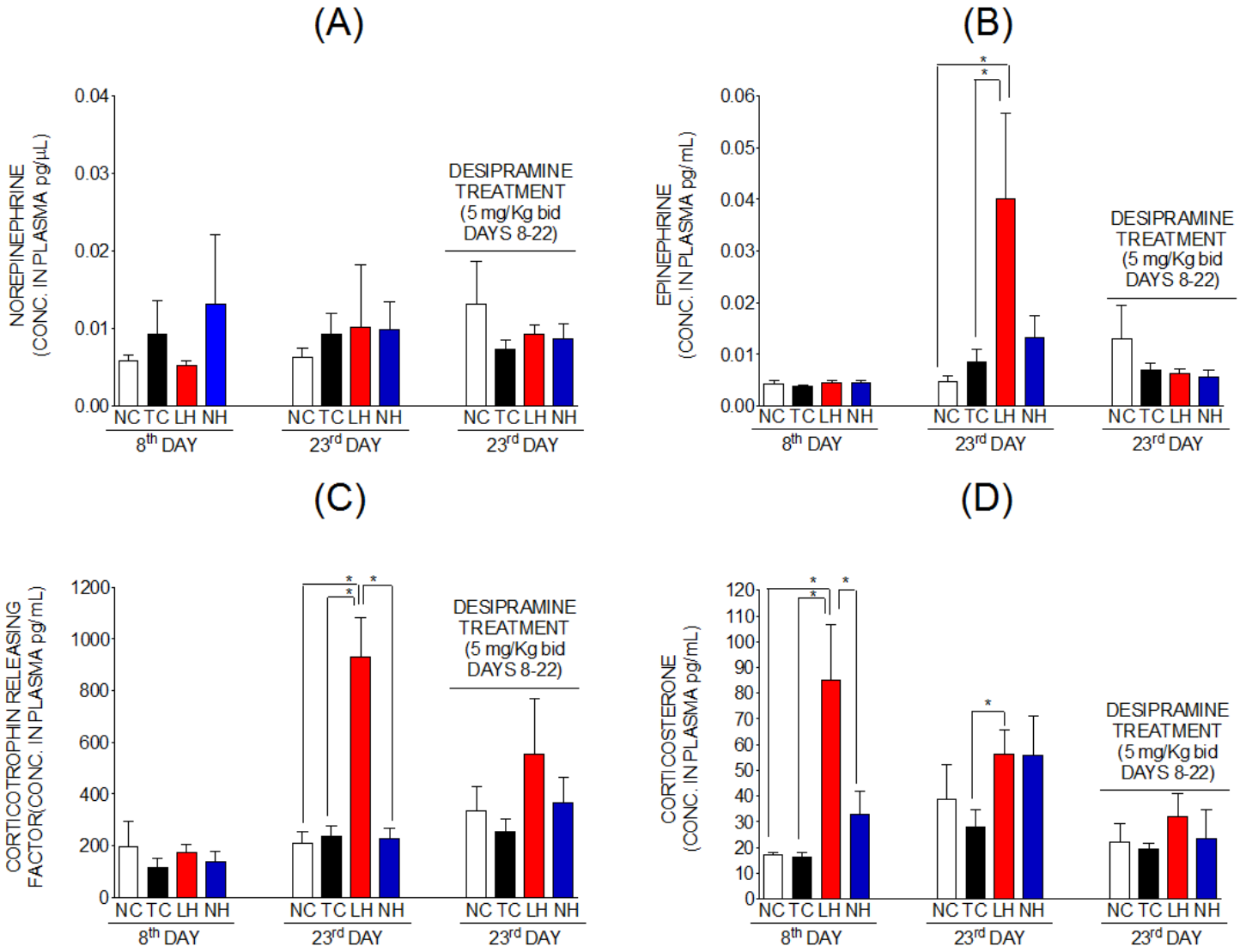

(A)
NOREPINEPHRINE (CONC. IN PLASMA pg/µL)
0.04
0.03
0.02
0.01
0.00
NC TC LH NH
8th DAY
NC TC LH NH
23rd DAY
DESIPRAMINE TREATMENT (5 mg/Kg bid DAYS 8-22)
NC TC LH NH
23rd DAY
(B)
EPINEPHRINE (CONC. IN PLASMA pg/mL)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
*
*
NC TC LH NH
8th DAY
NC TC LH NH
23rd DAY
DESIPRAMINE TREATMENT (5 mg/Kg bid DAYS 8-22)
NC TC LH NH
23rd DAY
(C)
CORTICOTROPHIN RELEASING FACTOR(CONC. IN PLASMA pg/mL)
1200
1000
800
600
400
200
0
*
*
*
NC TC LH NH
8th DAY
NC TC LH NH
23rd DAY
DESIPRAMINE TREATMENT (5 mg/Kg bid DAYS 8-22)
NC TC LH NH
23rd DAY
(D)
CORTICOSTERONE (CONC. IN PLASMA pg/mL)
120
110
100
90
80
70
60
50
40
30
20
10
0
*
*
*
*
NC TC LH NH
8th DAY
NC TC LH NH
23rd DAY
DESIPRAMINE TREATMENT (5 mg/Kg bid DAYS 8-22)
NC TC LH NH
23rd DAY

Figure 29: <u>Plasma stress hormone levels in repeatedly stressed rats exhibiting prolonged LH and NH behavior: With and without desipramine treatment</u>

Blood was collected from tail vein of each rat (control and repeatedly stressed) from each group on following time points a) Day 8 (before first escape testing) b) Day 23 (24h after second escape testing) c) Day 23 (24h after second escape testing) ;14 days of desipramine treatment from day8- day 22 .Plasma was separated from blood and measurements for stress hormones were done. Norepinephrine and epinephrine was measured using reverse phase high performance liquid chromatography equipped with coulometric electrochemical detector. CRF and corticosterone were measured using enzyme immunoassay kits

Panel (A) Norepinephrine, **Panel (B)** Epinephrine , **Panel (C)** Corticotrophin releasing factor, and **Panel (D)** Corticosterone levels in plasma of NC, TC, LH, and NH rats on day 8 and 23 post-stress (with and without 14 days of desipramine treatment). Data is expressed as Mean ± SEM, and * - mean pg/µL levels of stress hormones significantly different (LH vs. NH), (LH vs.TC), (LH vs. NC) ($p \leq 0.05$) n=5-9.

4.7 Comparison of brain biochemistry in naïve control and tested control rats

A single bout of restraint stress for TC might not be stressful but three bouts of restraint stress for TC has the potential to induce behavioral and biochemical changes. For this reason an additional control was added in the repeated stress study. The naive control (NC), which was not restrained or shocked, underwent the same handling and transportation procedures as TC and repeated stress rats. The single and repeated stress had differential effects on GRK2 protein levels. Therefore an additional receptor, histamine type 1 receptor (H_1-R) was measured in repeated stress studies: The H_1-R is preferentially regulated by GRK2. Thus, H_1-R levels will provide some indication of the specificity of receptor changes for receptors regulated by GRK3 vs. GRK2.

A comparison chart shows that NC and TC had no significant differences in protein/loading control ratios for GRK3, α_{2A}-AR, CRF_1-R, GRK2, TH, and Histamine receptors (H_1-R) in LC, amygdala and cortex (**Table 4)**. Therefore, in the subsequent sections data for LH and NH rats for changes in protein will be shown as % change of TC. This representation is similar to single stress data (where in TC was the only control).

	Locus Coeruleus (LC)			
	NC	TC	NC	TC
Desipramine Treatment	(-)	(-)	(+)	(+)
GRK3	0.34±0.03	0.36±0.02	0.35±0.05	0.39±0.02
α_{2A}-AR	0.32±0.02	0.36±0.03	0.34±0.03	0.34±0.03
CRF_1-R	0.25±0.03	0.26±0.02	0.21±0.02	0.27±0.04
GRK2	0.67±0.04	0.70±0.04	0.75±0.03	0.69±0.02
TH	0.63±0.07	0.70±0.06	0.60±0.03	0.72±0.04
H_1-R	0.33±0.02	0.35±0.02	0.35±0.05	0.32±0.05
	Amygdala			
	NC	TC	NC	TC
Desipramine Treatment	(-)	(-)	(+)	(+)
GRK3	0.32±0.02	0.32±0.02	0.33±0.03	0.36±0.04
α_{2A}-AR	0.34±0.02	0.32±0.02	0.39±0.04	0.39±0.03
CRF_1-R	0.23±0.02	0.22±0.02	0.22±0.02	0.26±0.02
GRK2	0.77±0.02	0.78±0.03	0.70±0.05	0.69±0.06
TH	0.34±0.02	0.35±0.04	0.38±0.03	0.34±0.05
H_1-R	0.43±0.02	0.39±0.03	0.37±0.05	0.44±0.04
	Cortex			
	NC	TC	NC	TC
Desipramine Treatment	(-)	(-)	(+)	(+)
GRK3	0.33±0.03	0.39±0.04	0.38±0.02	0.40±0.01
α_{2A}-AR	0.41±0.06	0.41±0.05	0.45±0.06	0.41±0.03
CRF_1-R	0.26±0.05	0.27±0.03	0.23±0.02	0.22±0.02
GRK2	1.16±0.07	1.20±0.05	1.05±0.89	1.07±0.07
TH	0.23±0.03	0.18±0.01	0.20±0.02	0.21±0.02
H_1-R	0.26±0.02	0.31±0.03	0.24±0.02	0.26±0.03

Table 4: Naïve and tested controls in repeated stress model shows similar protein levels in LC, amygdala, and cortex: With and without desipramine treatment

In repeated stress model on day 23 (24h after second escape testing), post-stress, rats were euthanized and brains were removed. LC, amygdala , and cortex were isolated from brains of NC, TC, LH and NH rats and levels of GRK3, α_{2A}-AR, CRF_1-R, GRK2, TH, H_1-R were measured using western blot technique. Values represented in table are MEAN ± SEM of immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios of naïve control (NC) and tested control (TC).

4.8 Effect of desipramine treatment on repeated stress-induced changes in GRK3, α_{2A}-AR, CRF_1-R, GRK2, TH, H_1-R levels

Twenty-four hours after the second escape test (confirming that LH and NH rats behave similarly as they did in first escape test trial on day 8), brains were harvested from euthanized rats. Levels of GRK3, α_{2A}-AR, CRF_1-R, GRK2, TH were measured as was done in the single stress study. Up to this point, only receptors (α_{2A}-AR, CRF_1-R**)** that were preferentially regulated by GRK3 were studied. Evidence suggests that the effects of repeated stress (3 bouts of tail shock stress) are different from the effects of single stress (one bout of tail shock stress). Therefore an additional receptor, histamine type 1 receptor (H_1-R) was preferentially regulated by GRK2 was also measured. Thus assessment of change in levels of H_1-R, if any, will provide some clarification of the preference of GRK3 vs. GRK2 for receptor regulation.

4.8.1 *Locus Coeruleus (LC)*

Repeated bouts of stress resulted in specific reduction of GRK3 associated with LH behavior and up-regulation of α_{2A}-AR and CRF_1-R. In addition to GRK3, reduction in GRK2 also is associated with LH behavior. Both GRK3 and GRK2 levels were reduced in the LC of LH rats (-38.2% and -27.5%; $p \leq 0.05$) compared to NH and TC rats (**Fig 30E)**. α_{2A}-AR levels were up-regulated in the LC of the LH rats (45.0%; $p \leq 0.05$) compared to NH and TC rats **(Fig**

30E).Similar to the α_{2A}-AR, 3 bouts of tail shock stress produced behaviorally specific changes in CRF_1-R levels in LC; CRF_1-R up-regulated in LH rats (38.4%; $p \leq 0.05$) but were unchanged in NH rats **(Fig 30E)**. This suggests that repeated stress results in defective down-regulation of α_{2A}-AR and CRF_1-R in LH rats (behavior specific change) mediated by GRK3. H_1-R levels were similar across the groups, suggesting that either alteration of GRK is not the primary regulatory mechanism for H_1-R or that H_1-R levels were not normally perturbed in stressful conditions. TH levels also were reduced in the LC (-25.4%; $p \leq 0.05$) of LH rats compared to TC rats (**Fig 30E)**.

A comparison between single and repeated stress-induced changes suggests that GRK3 is changed approximately to same extent by each condition. However GRK2 levels, which were unchanged in single stress, are reduced after repeated stress **(Table 5)**. This may indicate a cumulative effect on GRKs. Another important point is that α_{2A}-AR and CRF_1-R up-regulate in LH rats following repeated stress in contrast to the down-regulation noted in NH rats induced by single stress. However, in both single and repeated stress conditions LH rats have qualitatively higher levels of receptors **(Table 5)**. In single stress, levels of TH were increased in LH rats, which were in contrast to the repeated stress condition where reduction in TH levels was observed **(Table 5)**. The differential changes in levels of α_{2A}-AR, CRF_1-R GRK2, and TH may contribute to maintenance of LH behavior.

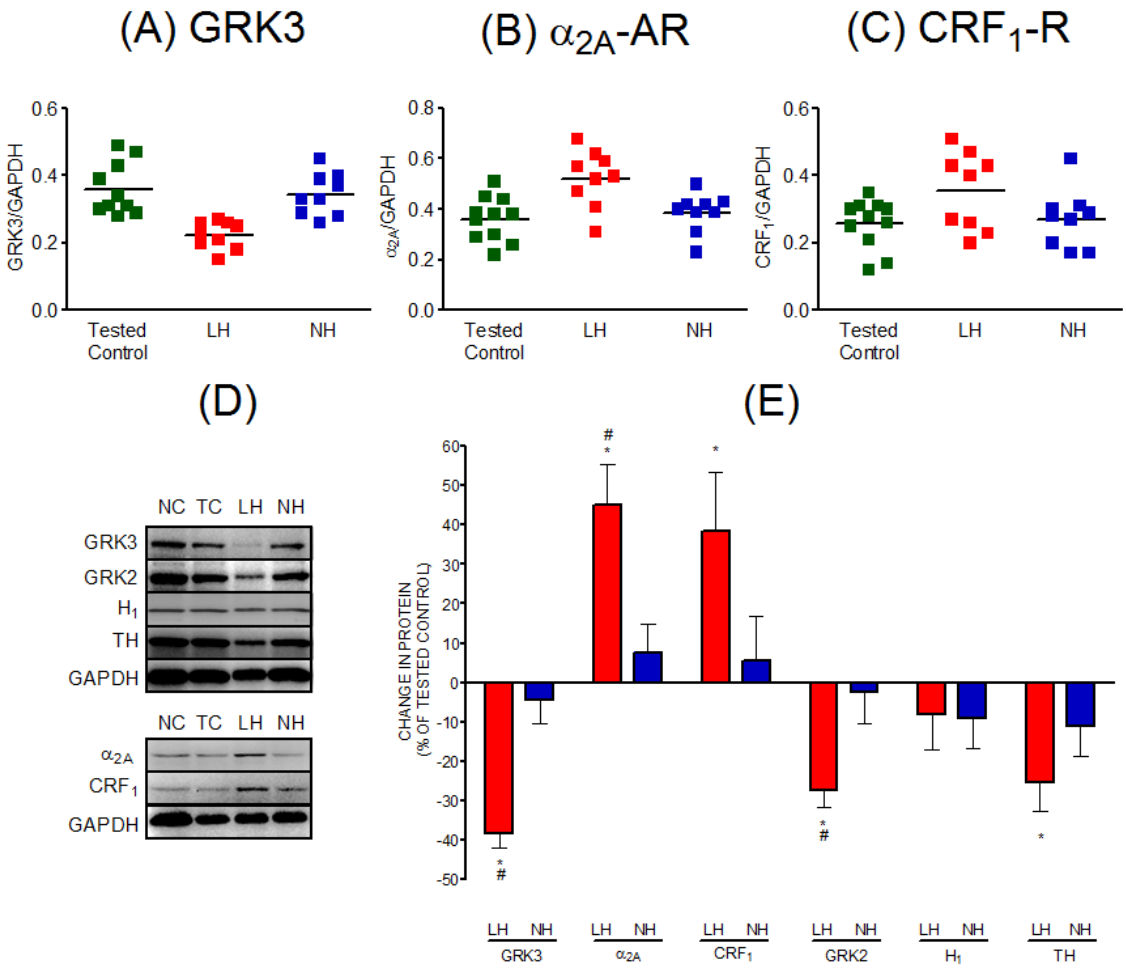

(A) GRK3
(B) α2A-AR
(C) CRF1-R
GRK3/GAPDH
α2A/GAPDH
CRF1/GAPDH
Tested Control
LH
NH
(D)
NC TC LH NH
GRK3
GRK2
H1
TH
GAPDH
α2A
CRF1
(E)
CHANGE IN PROTEIN (% OF TESTED CONTROL)
LH NH
GRK3
α2A
CRF1
GRK2
H1
TH

Figure 30: <u>Changes in biochemical parameter in LC-Day 23 post-stress in repeated stress model of prolonged LH behavior</u>

Rats were randomly separated into three groups. First group, naïve control (NC), was restrained did not receive repeated stress. Second group, TC, was restrained on days 1, 4, 7 but did not receive repeated stress. Third group received repeated stress (unpredictable, inescapable 100 tail shocks/ 1.2-2.0mA increased by 0.2mA/20shocks) on days 1, 4, 7. On day 8 and 22 escape latencies and escape failures were determined by escape testing for rats in each group. Day 8 escape latencies and escape failures data of repeated stress rats was subjected to K-means cluster analysis, which identified the presence of two populations within repeatedly stressed rats (LH-higher, NH-similar escape latencies/escape failures to TC or NC). Day 22 post-stress, rats exhibiting LH behavior on day 8 post-stress showed a similar level of escape latencies and escape failures to those recorded earlier. Day 23 post-stress (24h after escape testing or 15 days after last bout of TSS), rats were euthanized and brains were removed. LC was isolated from brains of TC, LH and NH rats and levels of GRK3, α_{2A}-AR, CRF_1-R, GRK2, H_1-R, and TH levels were measured using western blot technique. Mean immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R, GRK2, TH /Loading control) ratios were calculated followed by change in protein for LH, NH rats alone are calculated in comparison to tested control.

Panel (A, B, C) Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios of LH, NH rats in comparison to tested control. **Panel (D)** Representative western blots for GRK3, GRK2, H_1-R, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (E)**-Change in protein for LH and NH rats expressed as % of tested control. Significant change in protein levels ($p \leq 0.05$) *- (LH vs.TC), and # - (LH vs. NH) n=9-11.

Protein/%	Single stress (48h)		Repeated stress (Day 23)	
	LH	NH	LH	NH
GRK3	-28.1*	-6.9	-38.2*	-4.4
α_{2A}-AR	-6.5	-46.3*	45.0*	11.1
CRF_1-R	-3.6	-32.2*	38.4*	7.8
GRK2	-12.7	-2.4	-27.5*	-2.3
TH	27.2	1.2	-25.4*	-11.1

Table 5: Comparison of single and repeated stress-induced changes in GRK3, α_{2A}-AR, CRF_1-R, GRK2, and TH expressed as % of TC in LC

In repeated stress study , day 23 post-stress (24h after escape testing or 15 days after last bout of TSS), and in single stress study 48h post-stress (24h after escape testing) rats were euthanized and brains were removed. LC was isolated from brains of TC, LH and NH rats and levels of GRK3, α_{2A}-AR, CRF_1-R, GRK2, H_1-R, and TH levels were measured using western blot technique. Mean immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R, GRK2, TH /Loading control) ratios of LH, NH rats were calculated in comparison to tested control and change in protein for LH and NH rats expressed as % of tested control. Significant change in protein levels ($p \leq 0.05$) *- (LH, NH vs.TC)

4.8.2 Locus Coeruleus (LC-14 days of desipramine treatment)

Desipramine treatment (5mg/Kg bid for 14 days) normalized GRK3 levels in the LC (-38.2% significant to -15.8% non-significant) of repeated stress LH rats compared to TC rats **(Fig 31E, 32)**. Similarly to GRK3, GRK2 levels also were normalized by desipramine treatment in (-27.5% significant to -6.5 % non-significant) repeated stress LH rats compared to TC rats **(Fig 31E, 32)**. Restoration of GRK3 levels were associated with down-regulation of α_{2A}-AR in the LC (-31.7%; $p \leq 0.05$) of repeated stress LH rats compared to NH and TC **(Fig 31E, 32)**. However a direct effect of desipramine as a selective norepinephrine reuptake inhibitor (SNRI) causing an increase in norepinephrine within the synaptic cleft and consequent down-regulation of α_{2A}-AR cannot be ruled out. Similar to the changes in α_2-AR, CRF_1-R levels also were down-regulated (-23.4 % though not significant) by desipramine treatment in repeated stress LH rats **(Fig 31E)**. H_1-R levels were similar across the groups suggesting no change in receptor down-regulation was associated with reduced GRK2 levels in repeated stress LH rats. Desipramine treatment also restored TH levels (-8.9%) which were reduced in the LC (-25.4%; $p \leq 0.05$) of repeated stress LH rats compared to TC rats (**Fig 31E, 32)**.

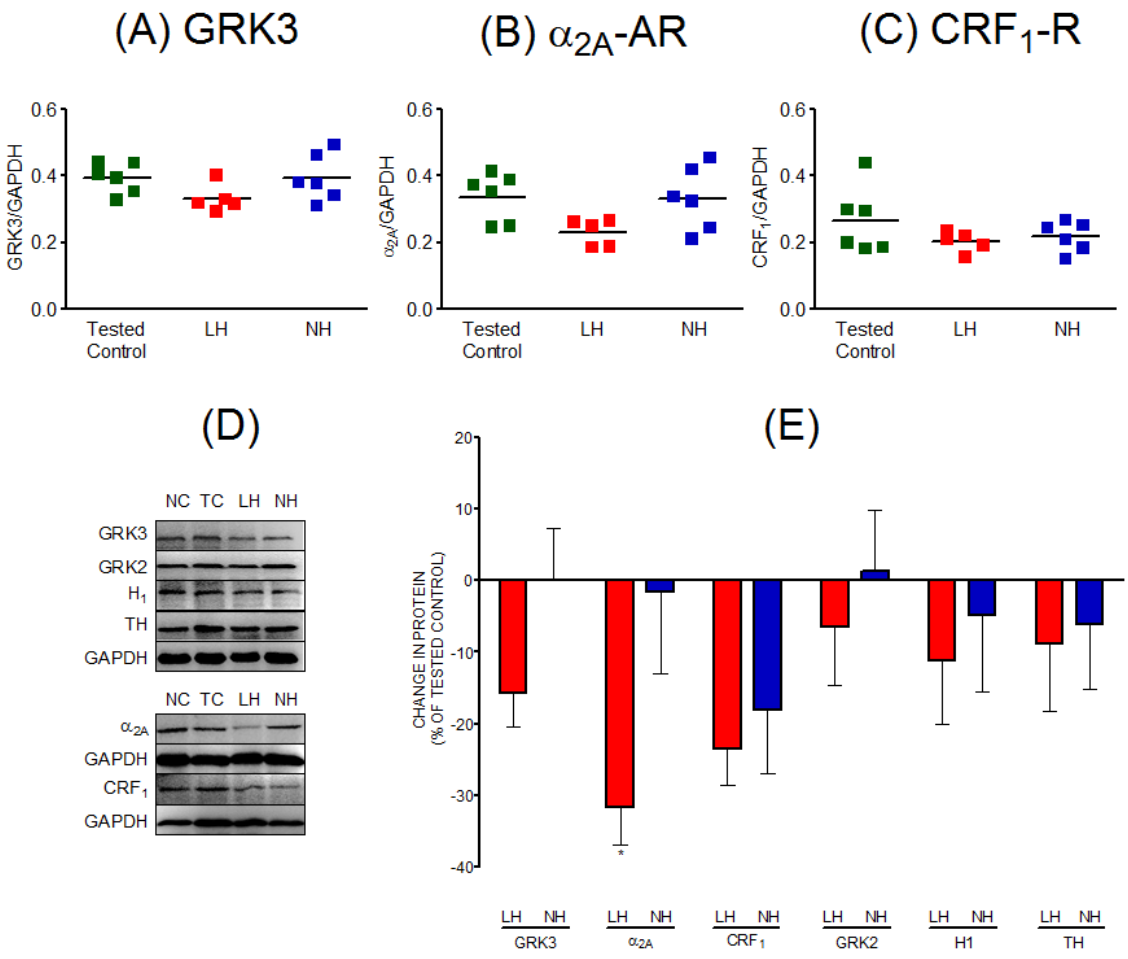

(A) GRK3
(B) α2A-AR
(C) CRF1-R
GRK3/GAPDH
α2A/GAPDH
CRF1/GAPDH
0.6
0.4
0.2
0.0
Tested Control
LH
NH
(D)
NC TC LH NH
GRK3
GRK2
H1
TH
GAPDH
α2A
CRF1
(E)
CHANGE IN PROTEIN (% OF TESTED CONTROL)
20
10
0
-10
-20
-30
-40
*
LH NH
GRK3
α2A
CRF1
GRK2
H1
TH

Figure 31: Normalization of repeated stress-induced changes in GRK3, GRK2, and TH along with reduction of α_{2A}-AR and CRF_1-R levels in LC by 14 days of desipramine treatment

Rats were randomly separated into three groups. First group, naïve control (NC), was restrained did not receive repeated stress. Second group, TC, was restrained on days 1, 4, 7 but did not receive repeated stress. Third group received repeated stress (unpredictable, inescapable 100 tail shocks/ 1.2-2.0mA increased by 0.2mA/20shocks) on days 1, 4, 7. On day 8 escape latencies and escape failures were determined by escape testing for rats in each group. Day 8 escape latencies and escape failures data of repeated stress rats was subjected to K-means cluster analysis, which identified the presence of two populations within repeatedly stressed rats (LH-higher, NH-similar escape latencies/escape failures to TC or NC). Fourteen days of desipramine treatment (5mg/kg, bid i.p.) from day 8 (after first escape testing)-day 22 (before second escape testing) was given to controls (NC/TC) and repeatedly stressed (LH/NH) rats. Day 23 post-stress (24h after second escape testing or 15 days after last bout of TSS), rats were euthanized and brains were removed. LC was isolated from brains of TC, LH and NH rats and levels of GRK3, α_{2A}-AR, CRF_1-R, GRK2, H_1-R, and TH levels were measured using western blot technique. Mean immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R, GRK2, TH /Loading control) ratios were calculated followed by change in protein for LH, NH rats alone are calculated in comparison to tested control.

Panel (A, B, C) Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios of LH, NH rats in comparison to tested control. **Panel (B)** Representative western blots for GRK3, GRK2, H_1-R, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (E)** Change in protein for LH and NH rats expressed as % of tested control. Significant change in protein levels ($p \leq 0.05$) *- (LH vs.TC) n=5-6.

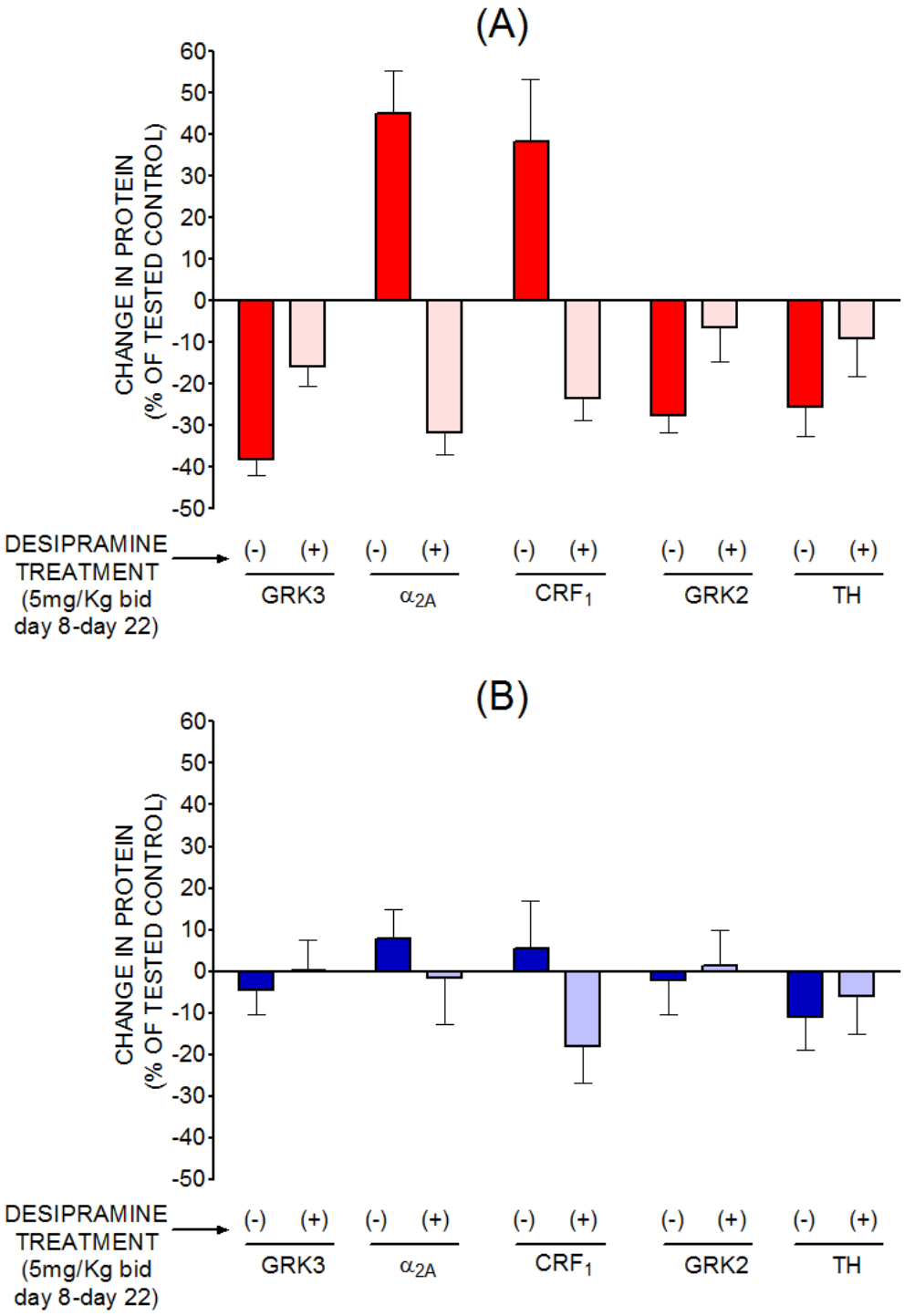

(A)
CHANGE IN PROTEIN
(% OF TESTED CONTROL)
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
DESIPRAMINE
TREATMENT
(5mg/Kg bid
day 8-day 22)
(-) (+) (-) (+) (-) (+) (-) (+) (-) (+)
GRK3
α2A
CRF1
GRK2
TH
(B)
CHANGE IN PROTEIN
(% OF TESTED CONTROL)
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
DESIPRAMINE
TREATMENT
(5mg/Kg bid
day 8-day 22)
(-) (+) (-) (+) (-) (+) (-) (+) (-) (+)
GRK3
α2A
CRF1
GRK2
TH

Figure 32: Comparison of repeated stress-induced changes in GRK3, α_{2A}-AR, CRF_1-R, GRK2, and TH levels in LH and NH rats in LC : With and without 14 days of desipramine treatment

Day 23 post-stress (24h after second escape testing or 15 days after last bout of TSS), rats were euthanized and brains were removed. LC was isolated from brains of NC, TC, LH and NH rats with and without desipramine treatment (14 days, 5mg/Kg bid from day8-day 22). Levels of GRK3, α_{2A}-AR, CRF_1-R, GRK2, H_1-R, and TH levels were measured using western blot technique **(For details refer to Fig 30, 31)**.

Panel (A) Changes in protein expressed as % of TC in LH rats.
Panel (B) Changes in protein expressed as % of TC in NH rats.

4.8.3 Amygdala

Similar to LC, amygdala also showed a repeated stress- induced behavioral specific reduction of GRK3 and up-regulation of α_{2A}-ARs associated with sustained LH behavior. GRK3 levels were reduced in the amygdala of LH rats (-25.3%; p≤0.05) compared to NH and TC rats (**Fig 33E)** while GRK2 levels which were significantly reduced in LC of LH rats in repeated stress were unchanged in amygdala compared to TC. Similarly to LC, α_{2A}-AR levels were up-regulated in the amygdala of the LH rats (29.6%; p≤0.05) compared to NH and TC **(Fig 33E)** rats**,** but there were no changes in CRF_1-R levels among LH, NH and TC rats . H_1-R and TH levels were similar across the groups (**Fig 33E)**.

A comparison between single and repeated stress-induced changes suggests that GRK3 is changed approximately to same extent (**Table 6)**. α_{2A}-AR shows relatively higher up-regulation in amygdala of LH rats under both single and repeated stress conditions, but repeated stress-induced changes in α_{2A}-AR can differentiate between LH and NH rats **(Table 6)**. In single stress, CRF_1-R levels were decreased in NH rats, but they were not changed in any of LH and NH rats after repeated stress, again suggesting that the effects of repeated stress are different from single stress.

Protein/%	Single stress (48h)		Repeated stress (Day 23)	
	LH	NH	LH	NH
GRK3	-21.0*	-7.1	-25.3*	-3.1
α_{2A}-AR	48.6*	25.2*	29.6*	-8.1
CRF_1-R	-8.2	-31.4*	-9.0	-13.0
GRK2	-11.1	0.7	-2.2	-8.7
TH	-8.4	-11.6	6.4	-1.2

Table 6: Comparison of single and repeated stress-induced changes in GRK3, α_{2A}-AR, CRF_1-R, GRK2, and TH expressed as % of TC in amygdala

In repeated stress study , day 23 post-stress (24h after escape testing or 15 days after last bout of TSS), and in single stress study 48h post-stress (24h after escape testing) rats were euthanized and brains were removed. Amygdala was isolated from brains of TC, LH and NH rats and levels of GRK3, α_{2A}-AR, CRF_1-R, GRK2, H_1-R, and TH levels were measured using western blot technique. Mean immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R, GRK2, TH /Loading control) ratios of LH, NH rats were calculated in comparison to tested control and change in protein for LH and NH rats expressed as % of tested control. Significant change in protein levels ($p \leq 0.05$) *- (LH, NH vs.TC)

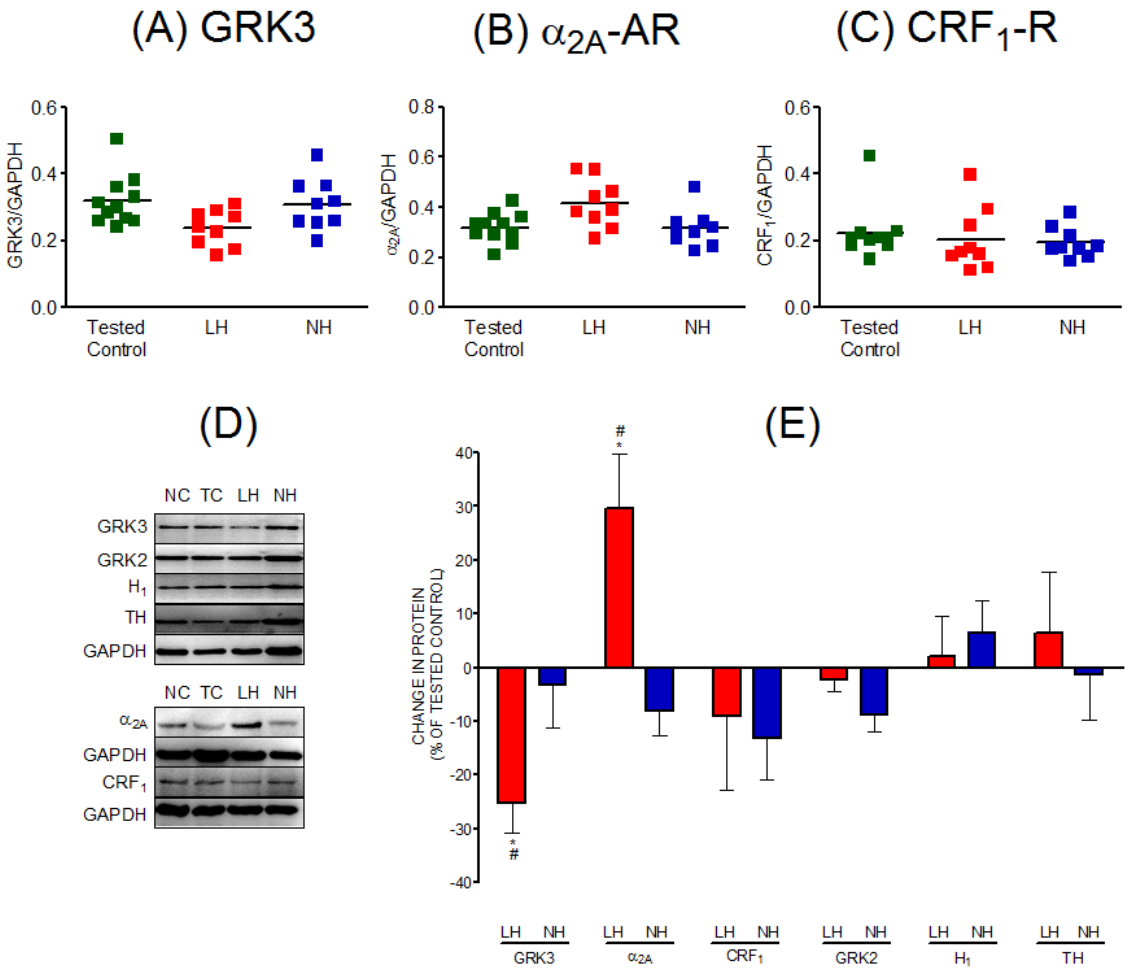

(A) GRK3
(B) α2A-AR
(C) CRF1-R
GRK3/GAPDH
α2A/GAPDH
CRF1/GAPDH
Tested Control
LH
NH
(D)
NC TC LH NH
GRK3
GRK2
H1
TH
GAPDH
α2A
CRF1
(E)
CHANGE IN PROTEIN (% OF TESTED CONTROL)
GRK3
α2A
CRF1
GRK2
H1
TH

Figure 33: Changes in GRK3 and α_{2A}-AR levels in amygdala-Day 23 post-stress in repeated stress model of prolonged LH behavior

Controls (NC /TC) and repeatedly stressed rats, day 23 post-stress (24h after second escape testing), were euthanized and brains were removed. Amygdala was isolated from brains of NC,TC, LH and NH rats and levels of GRK3, GRK2, TH , H_1-R , α_{2A}-AR and CRF_1-R were measured using western blot technique **(For details refer to Fig 30 legend)**.

Panel (A, B, C) Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios of LH, NH rats in comparison to tested control. **Panel (D)** Representative western blots for GRK3, GRK2, H_1-R, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (E)**-Change in protein for LH and NH rats expressed as % of tested control. Significant change in protein levels ($p \leq 0.05$) *- (LH vs.TC), and # - (LH vs. NH) n=9-11.

4.8.4 Amygdala (14 days of desipramine treatment)

Similar to LC, desipramine treatment (5mg/Kg bid for 14 days) normalized GRK3 levels in amygdala (-25.3% significant to -6.7% non-significant) of LH rats compared to TC rats **(Fig 34E, 35)**. Since GRK3 levels were normalized we see normalization of up-regulation of α_{2A}-AR in the amygdala of LH rats compared to NH and TC **(Fig 34E)**. CRF_1-R, GRK2, H_1-R, and TH levels did not change after desipramine treatment in amygdala of LH, NH and TC rats (**Fig 34E)**.

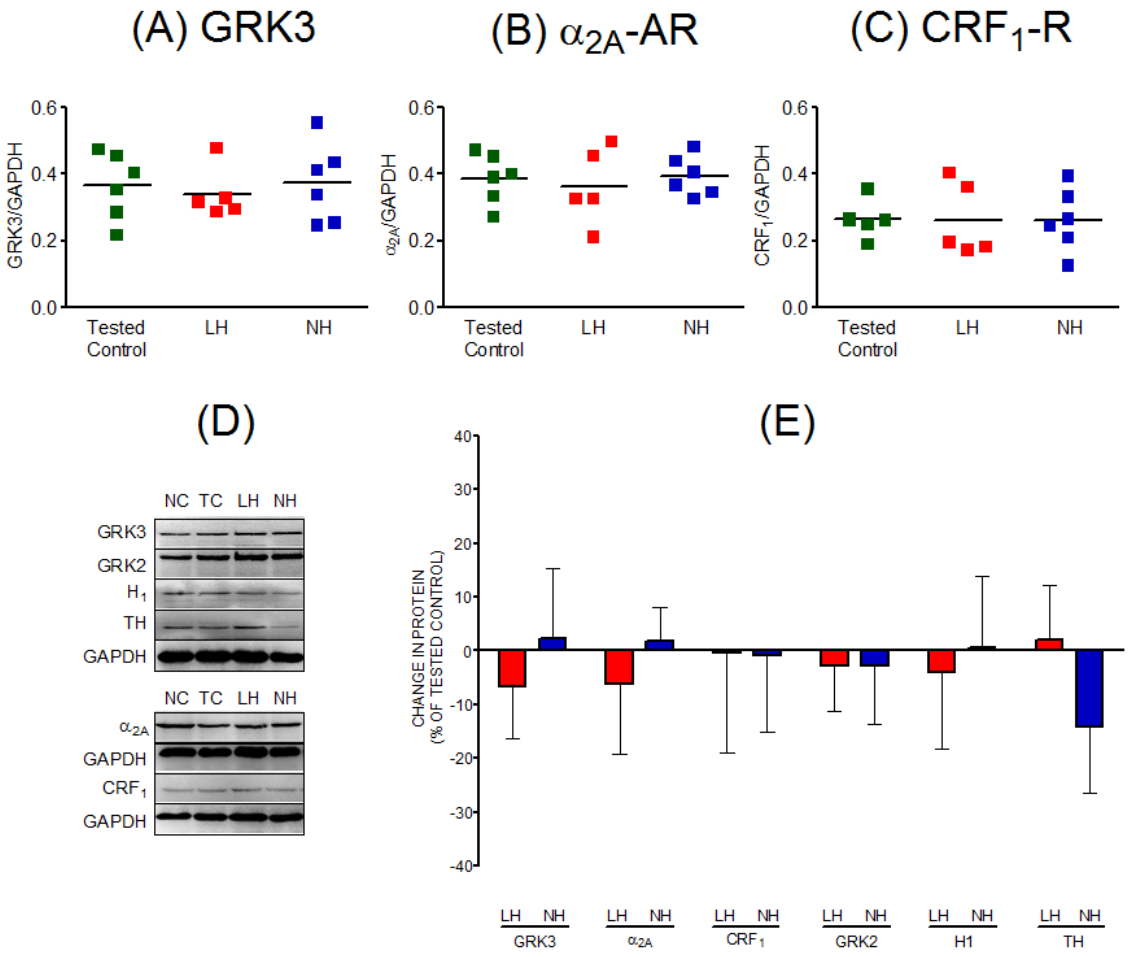
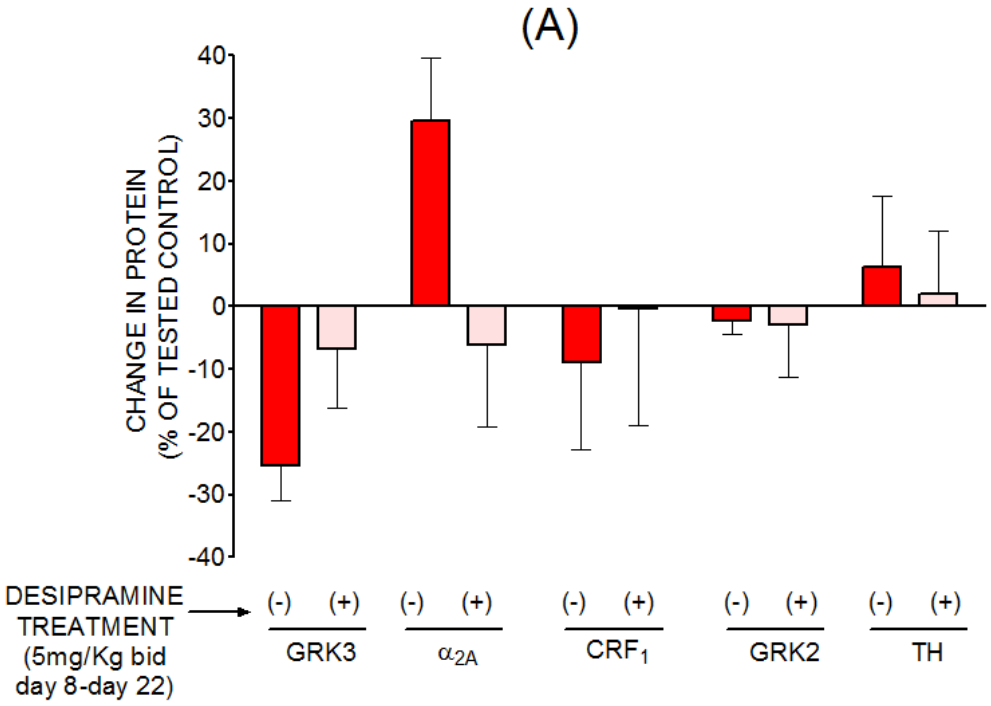

(A) GRK3
(B) α2A-AR
(C) CRF1-R
GRK3/GAPDH
α2A/GAPDH
CRF1/GAPDH
0.6
0.4
0.2
0.0
Tested Control
LH
NH
(D)
NC TC LH NH
GRK3
GRK2
H1
TH
GAPDH
α2A
CRF1
(E)
CHANGE IN PROTEIN (% OF TESTED CONTROL)
40
30
20
10
0
-10
-20
-30
-40
LH NH
GRK3
α2A
CRF1
GRK2
H1
TH

Figure 34: <u>Normalization of repeated stress-induced changes in GRK3 and α_{2A}-AR levels in amygdala by 14 days of desipramine treatment</u>
Fourteen days of desipramine treatment (5mg/kg, bid i.p.) from day 8 (after first escape testing)-day 22 (before second escape testing) was given to controls (NC/TC) and repeatedly stressed (LH/NH) rats. Day 23 post-stress (24h after second escape testing), rats were euthanized and brains were removed. Amygdala was isolated from brains of NC,TC, LH and NH rats and levels of GRK3, GRK2, TH , H_1-R , α_{2A}-AR and CRF_1-R were measured using western blot technique **(For details refer to Fig 31 legend)**.

Panel (A, B, C) Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios of LH, NH rats in comparison to tested control. **Panel (B)** Representative western blots for GRK3, GRK2, H_1-R, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (E)** Change in protein for LH and NH rats expressed as % of tested control, n=5-6.


(A)
CHANGE IN PROTEIN
(% OF TESTED CONTROL)
40
30
20
10
0
-10
-20
-30
-40
DESIPRAMINE
TREATMENT
(5mg/Kg bid
day 8-day 22)
(-) (+) (-) (+) (-) (+) (-) (+) (-) (+)
GRK3
α2A
CRF1
GRK2
TH

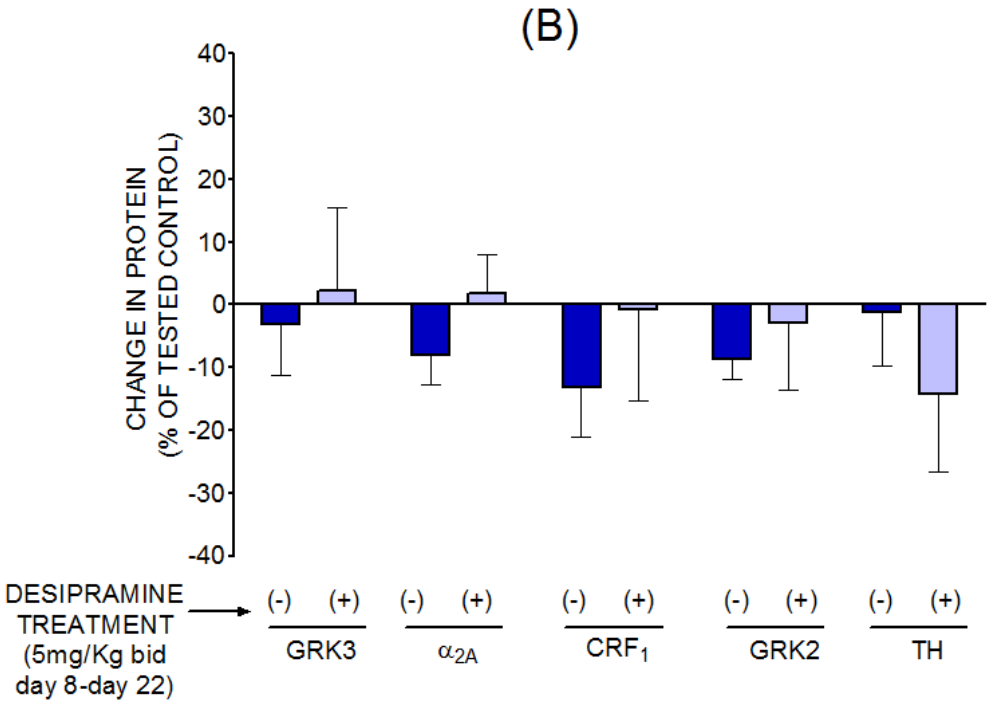

(B)
CHANGE IN PROTEIN
(% OF TESTED CONTROL)
40
30
20
10
0
-10
-20
-30
-40
DESIPRAMINE
TREATMENT
(5mg/Kg bid
day 8-day 22)
(-) (+) (-) (+) (-) (+) (-) (+) (-) (+)
GRK3
α2A
CRF1
GRK2
TH

Figure 35: <u>Comparison of repeated stress-induced changes in GRK3 and α_{2A}-AR levels in LH and NH rats in amygdala: With and without 14 days of desipramine treatment</u>

Day 23 post-stress (24h after second escape testing or 15 days after last bout of TSS), rats were euthanized and brains were removed. Amygdala was isolated from brains of NC, TC, LH and NH rats with and without desipramine treatment (14 days, 5mg/Kg bid from day8-day 22). Levels of GRK3, α_{2A}-AR, CRF_1-R, GRK2, H_1-R, and TH levels were measured using western blot technique **(For details refer to Fig 33, 34)**.

Panel (A) -Changes in protein expressed as % of TC in LH rats.
Panel (B) -Changes in protein expressed as % of TC in NH rats.

4.8.5 Cortex

Repeated stress did not change levels of GRK3, α_{2A}-AR, CRF_1-R, GRK2, H_1-R and TH proteins in cortex among LH, NH and TC rats (**Fig 36E)**. This is in contrast to single stress changes in cortex, where GRK3, GRK2 levels were reduced in both NH and LH rats as compared to TC accompanied by similar levels of changes in α_{2A}-AR and CRF_1-R in both NH and LH rats as compared to TC **(Table 7)**. The effects of single stress did not show any behavior specificity, suggesting stress mediated changes are global among stressed rats. The absence of change in protein levels in repeated stress in cortex further supports that effects of stress are no longer present. Moreover the data suggest that the behavioral consequence (prolonged LH behavior) of 3 bouts of tail shock stress (repeated stress) in LC and amygdala discriminate among LH and NH rats.

4.8.6 Cortex (14 days of desipramine treatment)

Repeated stress did not induce changes in GRK3, α_{2A}-AR, CRF_1-R, GRK2, H_1-R and TH proteins in cortex. This was not changed by desipramine treatment (5mg/Kg bid for 14 days) (**Fig 37E)**.

Single and repeated stress results from LC, amygdala, and cortex indeed suggest that these brain regions play distinct roles in the induction and maintenance of LH behavior by modulating levels of GRK3, GRK2, α_{2A}-AR, and CRF_1-R in a temporal and spatial context.

Protein/%	Single stress (48h)		Repeated Stress (Day 23)	
	LH	NH	LH	NH
GRK3	-29.8*	-33.2*	-3.6	3.3
α_{2A}-AR	28.8*	34.0*	-0.6	-11.8
CRF_1-R	-30.2*	-32.6*	-7.4	-15.7
GRK2	-16.4*	-17.9*	4.2	1.6
TH	-6.3	-14.4	12.0	-1.2

Table 7: Comparison of single and repeated stress- induced changes in GRK3, α_{2A}-AR, CRF_1-R, GRK2, and TH expressed as % of TC in cortex

In repeated stress study , day 23 post-stress (24h after escape testing or 15 days after last bout of TSS), and in single stress study 48h post-stress (24h after escape testing) rats were euthanized and brains were removed. Cortex was isolated from brains of TC, LH and NH rats and levels of GRK3, α_{2A}-AR, CRF_1-R, GRK2, H_1-R, and TH levels were measured using western blot technique. Mean immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R, GRK2, TH /Loading control) ratios of LH, NH rats were calculated in comparison to tested control and change in protein for LH and NH rats expressed as % of tested control. Significant change in protein levels ($p \leq 0.05$) *- (LH, NH vs.TC)

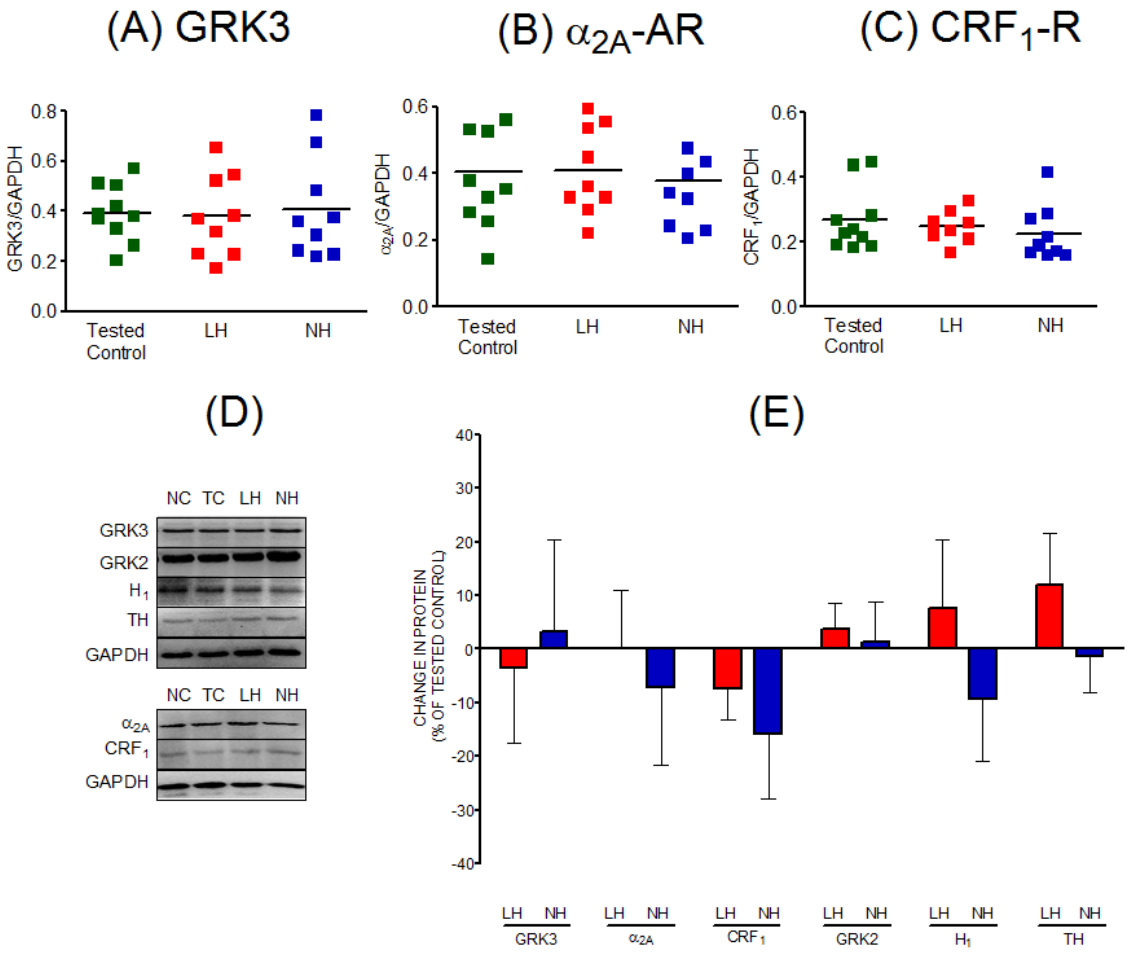

(A) GRK3
(B) α2A-AR
(C) CRF1-R
GRK3/GAPDH
α2A/GAPDH
CRF1/GAPDH
0.0
0.2
0.4
0.6
0.8
Tested Control
LH
NH
(D)
NC TC LH NH
GRK3
GRK2
H1
TH
GAPDH
α2A
CRF1
(E)
CHANGE IN PROTEIN (% OF TESTED CONTROL)
40
30
20
10
0
-10
-20
-30
-40
LH NH
GRK3
α2A
CRF1
GRK2
H1
TH

Figure 36: No change in GRK3, α_{2A}-AR, CRF_1-R, GRK2, H_1-R and TH levels in cortex- Day 23 post-stress in repeated stress model of prolonged LH behavior

Controls (NC /TC) and repeatedly stressed rats, day 23 post-stress (24h after second escape testing), were euthanized and brains were removed. Cortex was isolated from brains of NC,TC, LH and NH rats and levels of GRK3, GRK2, TH , H_1-R , α_{2A}-AR and CRF_1-R were measured using western blot technique **(For details refer to Fig 30 legend)**.

Panel (A, B, C) Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios of LH, NH rats in comparison to tested control. **Panel (D)** Representative western blots for GRK3, GRK2, H_1-R, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (E)**-Change in protein for LH and NH rats expressed as % of tested control, n=9-11.

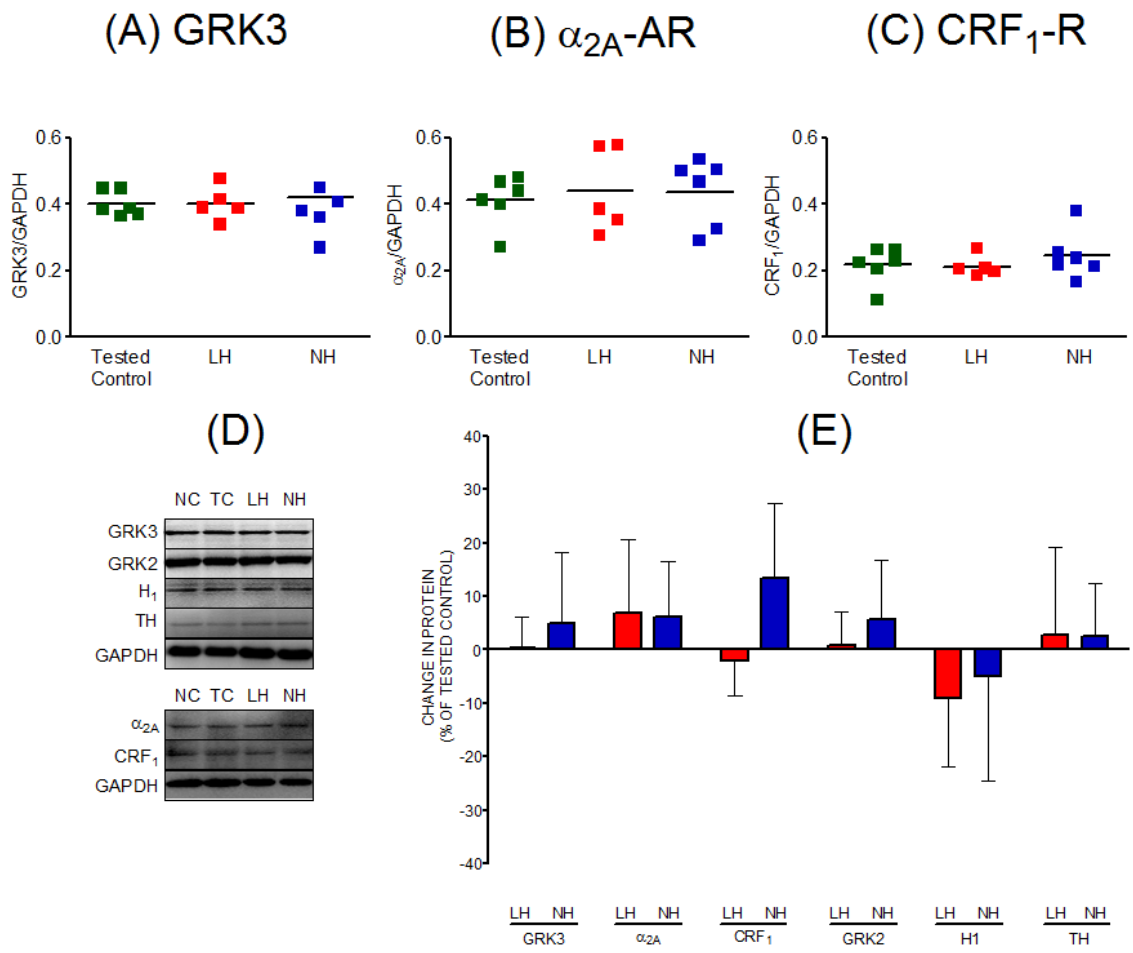

(A) GRK3
(B) α2A-AR
(C) CRF1-R
GRK3/GAPDH
α2A/GAPDH
CRF1/GAPDH
0.6
0.4
0.2
0.0
Tested Control
LH
NH
(D)
NC TC LH NH
GRK3
GRK2
H1
TH
GAPDH
α2A
CRF1
(E)
CHANGE IN PROTEIN (% OF TESTED CONTROL)
40
30
20
10
0
-10
-20
-30
-40
LH NH
GRK3
α2A
CRF1
GRK2
H1
TH

Figure 37: No change in GRK3, α_{2A}-AR, CRF_1-R, GRK2, H_1-R and TH levels in cortex by 14 days of desipramine treatment

Fourteen days of desipramine treatment (5mg/kg, bid i.p.) from day 8 (after first escape testing)-day 22 (before second escape testing) was given to controls (NC/TC) and repeatedly stressed (LH/NH) rats. Day 23 post-stress (24h after second escape testing), rats were euthanized and brains were removed. Cortex was isolated from brains of NC,TC, LH and NH rats and levels of GRK3, GRK2, TH , H_1-R , α_{2A}-AR and CRF_1-R were measured using western blot technique **(For details refer to Fig 31 legend)**.

Panel (A, B, C) Immunoreactive band intensity (GRK3, α_{2A}-AR, CRF_1-R /Loading control) ratios of LH, NH rats in comparison to tested control. **Panel (B)** Representative western blots for GRK3, GRK2, H_1-R, TH, α_{2A}-AR, CRF_1-R, and loading controls. **Panel (E)** Change in protein for LH and NH rats expressed as % of tested control, n=5-6.

5. DISCUSSION

The present study identified reduced GRK3 levels and associated impaired regulation of α_{2A}-AR and CRF_1- R levels as a discriminator for stress susceptible populations from stress resilient individuals in conditions of single and repeated stress. This conclusion is supported by data including: temporal and spatial correlates of α_{2A}-AR/CRF_1-R and GRK3 levels traversing from appearance to disappearance of stress susceptible LH behavior induced by single stress, differences at the level of α_{2A}-AR/CRF_1-R and GRK3 between single and repeated stress, demonstrated by developing a new validated model for studying prolonged LH behavior induced by repeated stress, and using pharmacological intervention (desipramine treatment) for reversal of behavioral changes induced by repeated stress which was correlated with restoration of GRK3 levels and associated α_{2A}-AR/CRF_1-R levels.

The study involved utilization of the learned helpless (LH) paradigm, which is used as an animal model to screen antidepressant drugs. This model has an added advantage of identifying a stress resilient (Non-helpless-NH) population as well as a stress susceptible population (Learned helpless-LH) amongst a group of stressed rats. The stress resilient population is largely overlooked. Most of the research has focused on LH animals which showed defects in serotonergic, noradrenergic and CRF related neuronal regulation [23,

85, 90-92]. Since our focus was on GRK3, and its target receptors, α_{2A}-AR/CRF_1-R and their association with stress susceptibility, we concentrated on the brain regions involved in NE/CRF neuroanatomical circuit: LC (primary noradrenergic nuclei) and amygdala (expresses abundant CRF_1-Rs). In order to determine what other major stress responsive brain regions contribute to changes in GRK3, α_{2A}-AR and CRF_1-R, preliminary studies were conducted to identify differences between brains of LH and NH rats extracted 48h-post stress (24h after the escape task). Brain regions tested included LC, amygdala, hypothalamus, hippocampus, cortex, and striatum along with a region that shows little involvement with the stress response; the cerebellum (Appendix).

The regions which showed significant detectable differences in all three proteins of interest GRK3, α_{2A}-AR, and CRF_1- R, between LH and NH rats with respect to control were LC, amygdala and cortex. Though LC and amygdala showed behavioral specific changes in GRK3, α_{2A}-AR, and CRF_1- R, cortex showed differences that were generalized to both LH and NH rats as compared to controls. Thus alterations appear to be more stress specific in the cortex as opposed to the behavioral specific changes seen for LC and amygdala. Secondly, 48h post-stress data allowed for retrospective analysis of which proteins could be markers for predicting the behavior of the rats.

5.1 Single stress induces behavioral specific changes in GRK3, α_{2A}-AR and CRF_1 levels

5.1.1 48h post-stress: Biochemical alterations correlate with behavior

Reduced levels of GRK3 in LC and amygdala are associated with learned helplessness in a sub-population of rats subjected to single stress, whereas normal GRK3 levels are observed in these brain areas in NH rats. Moreover, reduced levels of GRK3 are associated with differences in the regulation of α_{2A}-AR and CRF_1-R in LC and amygdala of LH and NH rats. Cortex, in contrast, showed reduction in GRK3 along with changes in α_{2A}-AR and CRF_1-R in both LH and NH rats as compared to control; suggesting changes here are stress specific, affecting LH and NH rats equally. In addition, these changes in GRK3, α_{2A}-AR and CRF_1 in LC and amygdala but not cortex can be used to independently identify stress resilient NH and non-resilient LH populations within a group of rats following exposure of single stress.

Cluster analysis using the levels of GRK3, α_2-AR and CRF_1-R individually as variables suggested that, of the regions studied, changes in the LC are most predictive of the behavioral effect of single stress. In cortex 59, 53, and 53% overlap was observed between the populations identified by cluster analysis for GRK3, α_{2A}-AR and CRF_1-R, respectively, and the groups identified behaviorally as LH and NH rats. Given that 50% overlap would occur randomly, changes in

cortex appear to not be related to LH vs. NH behavior. Rather, the effects in cortex appear generally related only to stress exposure. In amygdala, 82, 71 and 77% overlap was observed between the groups identified by cluster analysis for GRK3, α_{2A}-AR and CRF_1-R, respectively, and the LH and NH groups. This overlap is considerably lower than the 94, 92, and 92% overlap for GRK3, α_{2A}-AR and CRF_1-R respectively that was observed in LC. Cluster analysis using levels of GRK3, α_{2A}-AR and CRF_1-R simultaneously as a combined variable suggests that, LC showed 100% agreement, amygdala showed 83% overlap and cortex showed 59% overlap with the behavioral data. This indicates that the changes in GRK3, α_{2A}-AR and CRF_1-R in LC are most closely associated with the development of LH or NH behavior after single stress, though a contribution of amygdala cannot be denied either.

It is known that it takes 24h post-stress for the escape task to identify distinct LH and NH behavior [23-25, 88] but the question was how to study the development of behavior and corresponding changes in proteins if the behavior cannot be identified until 24h post-stress. In addition, LH rats, as a result of prolonged escape latency relative to NH rats after single stress, receive approximately 4 times as many shocks as NH rats during escape testing in the shuttle box. Therefore, it is possible that differences in levels of GRK3, α_{2A}-AR and CRF_1-R after single stress and escape testing are an artifact of shuttle box testing, and not a true consequence of single stress leading to differences in

escape latency. To address these questions cluster analysis approach was used and the question was asked, can changes in GRK3, α_{2A}-AR and CRF_1-R levels in LC and amygdala predict differentiation of stressed animals into two groups after single stress exposure before behavioral testing? There was valid reason to use this approach, as changes in GRK3, α_{2A}-AR and CRF_1-R levels in LC and amygdala we can identify the same bimodal population 48h post stress as was identified by the escape task 24h post-stress.

5.1.2 Temporal correlates of changes in GRK3, α_2-AR and CRF_1 levels with the development of LH behavior

At 24h post-stress, the time when escape testing was performed, single stress rats can be segregated into two populations based upon K-means cluster analysis of the collective GRK3, α_{2A}-AR and CRF_1-R data in LC. Furthermore, the data in these clusters appear very similar to the 48h post-stress data in the LH and NH population identified by escape testing. This suggests that shuttle box testing was not a confounder and did not produce artifactual differences in 48h post-stress biochemical data. Moreover changes in GRK3, α_{2A}-AR and CRF_1-R levels present at 24h post-stress might be responsible for the single stressed rats exhibiting distinct LH and NH behavior.

To determine how early changes in GRK3, α_{2A}-AR and CRF_1-R levels show up, studies at 1h post-stress were done. Results suggest that similar to

24h post-stress, 1h post-stress rats can be clustered into two significantly different populations (C1 and C2) using GRK3, α_{2A}-AR and CRF_1-R levels as variables for K-means cluster analysis. For both 1h and 24h post-stress one population (cluster 2, C2→LH like) exhibited significantly reduced GRK3 along with increased α_{2A}-AR and CRF_1-R levels in LC while the other group (cluster 1, C1→NH like) showed no change in GRK3 at 1h post-stress and a slight increase of GRK3 along with reductions in α_{2A}-AR and CRF_1-R levels in LC at 24h post-stress **(Table 7)**. These changes suggest that in LC, as early as 1h post-stress, biochemical discrimination starts which set the stage for behavioral discrimination of stressed animals in the escape latency task at 24h post-stress. The time course of these changes tracks with 48h post-stress changes in LC of LH and NH rats, because GRK3, α_{2A}-AR and CRF_1-R levels of cluster C2 are in agreement with LH rats and cluster C1 with NH rats.

It appears from the data that stress-induced selective changes are initiated by LC and that it takes time for the change in LC to affect the amygdala. At 1h post-stress, levels of proteins (GRK3, α_{2A}-AR and CRF_1-R) in amygdala were unable to cluster stressed rats to two sub-populations while there were significant differences between stressed and control rats. Evidence suggests that the LC- noradrenergic system is one of the most important stress responsive regions which provide the platform for integration of emotional and cognitive responses to stress-mediated events [103]. Among the projections from LC to

other brain regions, the interconnection with amygdala is unique as it provides feedback reciprocal projections (both afferent and efferent) which can regulate each other's function [8, 12]. Outside the olfactory region, the LC is the area that exhibits highest expression of GRK3 [16]. LC is the primary noradrenergic nuclei in brain along with abundant CRF_1-R expression. This might explain why changes at the levels of GRK3 and associated α_{2A}-AR and CRF_1-R changes start as early as 1h post-stress and then when it starts affecting amygdala, the system reaches a steady state. Therefore 24h post-stress, K-means cluster analysis of levels of protein (GRK3, α_{2A}-AR and CRF_1-R) in amygdala identify two populations among stressed rats. One of the populations (cluster 2 C2→LH like) show reduced GRK3 levels accompanied by relative less down regulation of receptors. This data also is in agreement with 48h post stress data of amygdala **(Table 8)**.

If the changes in LC and amygdala have any functional connections, then the population identified on the basis of cluster analysis in these regions should have some symmetry. At 1h post-stress, LC shows distinction of stressed rats into two populations; however amygdala showed no population discrimination on the basis of GRK3, α_{2A}-AR and CRF_1-R levels. At 24h post-stress, LC and amygdala both show stressed rats could be distinguished into two populations by cluster analysis. When the populations identified by cluster analysis

independently in LC and amygdala were matched, 71.4% symmetry was observed (**Fig 38)**.

The cluster symmetry suggests that changes in GRK3, α_{2A}-AR and CRF_1-R in LC are associated with amygdala at 24 h post-stress. Furthermore, changes in LC occur earlier and therefore one might speculate that the activity of LC derived projections to amygdala takes some time to induce changes in amygdala, thus causing differentiation of amygdala data into two populations based on cluster analysis of GRK3, α_{2A}-AR and CRF_1-R levels at the 24 h but not at the 1h post stress time point.

In cortex, at 48h post- stress no behaviorally specific changes in GRK3, α_{2A}-AR and CRF_1-R were observed. GRK3, α_{2A}-AR and CRF_1-R levels changed to similar extent in both LH and NH animals suggesting global stress specific changes. Cluster analysis of GRK3, α_{2A}-AR and CRF_1-R individually or collectively in cortex without consideration of behavior also revealed the same results. In cortex, cluster analysis of GRK3, α_{2A}-AR and CRF_1-R did not identify two populations unlike in LC and amygdala. The data from LC and amygdala compared to cortex thus suggested a regional specificity of behavioral specific change. To confirm this conclusion cortex GRK3, α_{2A}-AR and CRF_1-R levels in stressed rats were sorted according to population (cluster C1 and C2) identified using the LC data. 24h post-stress the C1 and C2 populations of cortex showed similar changes in GRK3, α_{2A}-AR and CRF_1-R levels**,** in agreement with results

from cortex 48h post-stress. In contrast, cortex 1h-post stress data sorted on the basis of LC clusters showed that cluster C1 has reduced GRK3 levels and associated up-regulation of α_{2A}-AR and CRF_1-R. This might suggest that changes occurring in cortex at 1h might have association with changes occurring in LC. Literature suggests that perception of stress is the key in determining whether a stressor is stressful or non-stressful. Effects of stress being translated into behavioral change require a stressor to be perceived as a stressful event. Cortex plays a major role in perception of stress and associated resilience, if the stress is perceived non-stressful [104, 105]. It can be speculated that the cortical functions responsible for perceiving a stressor as stressful might be regulated by noradrenergic inputs from the LC.

The suggestion that LC plays a critical role in LH and stress susceptibility is compatible with a significant body of literature. First, LH is the result of the application of inescapable unpredictable stress and inescapable stress results in marked activation of the LC [106]. Second, administration of α_2-AR agonists into the LC can reverse the behavioral effects of exposure to inescapable stress [92]. Third, intra-LC administration of α_2-AR antagonists produces behavioral effects that mimic those of inescapable stress [93]. Fourth, during stress increased LC firing increases dorsal raphe nucleus (DRN) 5-hydroxy tryptamine (5-HT) neuronal activation [94, 95] . DRN hyper-activation by LC's noradrenergic projections might involve activation of alpha$_1$-AR (α_1-AR) located on DRN

neurons which might contribute to development of LH behavior. Intra-DRN administration of α_1-AR antagonist only before the unpredictable and inescapable stress prevented the escape deficits in rats, but it could not reverse these deficits when given before escape testing [96].

The fact that inhibitory α_{2A}-ARs are down-regulated in the LC of resilient NH rats appears inconsistent with the suggestive evidences from literature. However, α_{2A}-ARs in LC are present prejunctionally on recurrent collateral neurons and inhibit the release of NE onto LC neurons, and postjunctionally on LC neurons where they inhibit neuronal firing. Reduced prejunctional inhibition would increase NE release and enhance NE inhibition of the LC, while reduced postjunctional inhibition would reduce NE inhibition of LC. Down-regulation of both α_{2A}-AR and CRF_1-R was observed only in the stress resilient NH rats that do not develop LH. In rats that developed LH, GRK3 levels were reduced and there were no α_{2A}-AR and CRF_1-R changes compared to TC rats. It is tempting to speculate that the changes in α_{2A}-AR and CRF_1-R in NH rats contribute to a net decrease in LC responsiveness, preventing the hyper-responsiveness of the LC that is characteristic of LH rats. Direct assessment of neuronal responses to α_{2A}-AR and CRF_1-R alone and in combination is required to determine the precise consequences of the changes in receptors to the firing of LC neurons. Although there are studies which have used c-FOS as a marker of differential regional neuronal activation in hippocampus, hypothalamus and lateral septum

[107, 108] of LH and NH rats, but this information is still missing for the LC. Therefore measurement of c-FOS reactivity also can provide an index of differential neuronal activation responses in LC of identified LH and NH populations from escape testing (24h post-tress). Finally, chronic treatment with many currently employed antidepressants agents reduces the activity of LC neurons [93, 109, 110]. A role of the LC in determining stress resilience also has been suggested by work related to gender and stress. For example, Valentino and co-workers recently reported that sex differences in the regulation of CRF_1 receptor signaling in LC may play a role in female vulnerability to stress-related psychopathology [111]. This group also has suggested that CRF in LC may contribute to the role of stress in vulnerability to opiate abuse [40, 112]. Therefore, the results showing temporal changes in the development of LH behavior add to the evidence supporting a role for the LC in the development of psychopathology as a result of stress.

The amygdala and its role in development and sustenance of LH behavior have primarily been studied from the perspective of the model wherein the initial stress and the escape task are done in same environment (context similarity). In this model rat's show escape deficits for 42 days post-stress [85, 100]. The escape deficits depend on conditioned fear and involvement of the basolateral amygdala is identified as lesions in this region reverse the escape deficit [86].

		LOCUS COERULEUS			AMYGDALA		
Post Stress	**Identified Groups**	**GRK3**	**α_{2A}-AR**	**CRF_1-R**	**GRK3**	**α_{2A}-AR**	**CRF_1-R**
1 hr	**Cluster 1**	↔	↔	↔	No cluster formation		
1 hr	**Cluster 2**	↓↓	↑	↑			
24 hr	**Cluster 1**	↑	↓	↓	↔	↓↓	↓↓
24 hr	**Cluster 2**	↓↓	↑	↑	↓↓	↔	↔
48 hr	**NH**	↔	↓↓	↓↓	↔	↑	↓
48 hr	**LH**	↓↓	↔	↔	↓↓	↑↑	↔

Table 8: Single stress-induced time dependent changes in GRK3, α_{2A}-AR, and CRF_1-R in LC and amygdala for LH and NH behavior

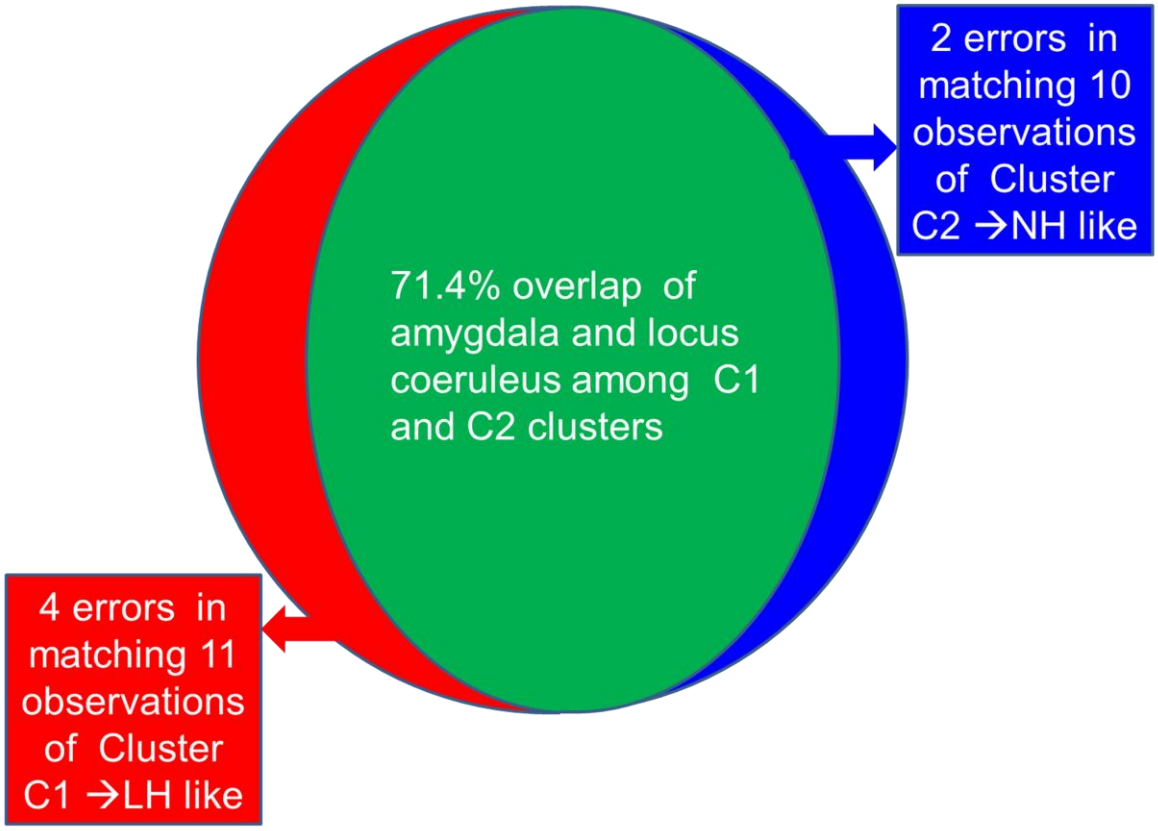


Figure 38: <u>Cluster symmetry between amygdala locus and coeruleus 24h post-stress plotted as venn diagram</u>
Cluster symmetry estimated by matching every observation between amygdala and locus coeruleus for C1 and C2 clusters. Number of errors in matching of C1 and C2 cluster was determined. % overlap was calculated by taking total errors (2 in C1 +4 in C2 =6) divided by total number of compared observations (n=21).

In the model where the stress and escape testing are done in different environments (the model utilized in this study), there is no fear transferred from the stress context to the escape task context. Evidence also suggests that in this model, shuttle box escape deficits induced by uncontrollable stress are independent of fear. Interventions that have the capacity to eliminate fear during escape testing, (benzodiazepines or electrolytic lesions of the amygdala) do not interfere with escape latencies and escape failures 24 h following single stress [31, 87, 88]. Thus the role of the amygdala in single stress-induced LH behavior in our model is more indirect and depends on its interaction with LC. This conclusion is supported by the data from amygdala, where 24h after stress was required before distinctions into two clusters based on GRK3, α_{2A}-AR, and CRF_1 levels were observed, while these distinctions occur as early as 1h post-stress in LC.

The LC-noradrenergic activation of the amygdala and a reciprocal activation of LC by CRF innervation from amygdala [8, 12] appear to form a feed forward mechanism. When escape testing is done, it acts as a milder stress sufficient to cause sensitization of stressed rats. It is posited that in LH rats, GRK3 depletion during stress prevents desensitization of the CRF_1-R and thus sensitization to future milder stress (escape testing) is exaggerated. Down regulation of CRF_1-R in NH rats inhibits the effect of the feed forward mechanism on the subsequent stress response escape testing. Thus the

sensitization response to milder subsequent stress in shuttle box is decreased by CRF_1-R down-regulation in NH rats which allows them to behave normally while LC of LH rats respond at a heightened level due to lack of CRF_1-R down-regulation.

Depletion of GRK3 and the accompanying changes in α_{2A}-AR and CRF_1-R regulation in the LC in LH rats support a role of GRK3 as a regulator of stress resilience in LC. GRK3 modulates receptor signaling by catalyzing the agonist-induced phosphorylation of receptors, facilitating the recruitment of arrestin to the receptor. This process initiates G protein-mediated signaling desensitization [113, 114]. CRF_1 receptors activate LC neurons, increasing neuronal firing, whereas α_2-AR inhibits the firing of LC neurons [115, 116]. Therefore, depletion of GRK3 in LC would reduce the capacity of GRK3 to desensitize the signaling of both an excitatory and an inhibitory input to the LC. Because hyper-responsiveness of the LC neurons is associated with the behavioral consequences of inescapable stress, it is suggested that rats resistant to LH would be able to make adaptive changes to limit LC hyper-responsiveness. This could be accomplished by either reducing the response to stimulatory inputs by CRF_1-R or by enhancing the response to inhibitory inputs via the α_{2A}-ARs.

The suggestion that reduced levels of GRK3 after single stress should be associated with the negative consequences of stress is not universally supported by the literature. For example, compared to wild type mice, GRK3 knockout mice

exhibit reduced immobility, or reduced depression-like behavior after forced swim [75]. This effect of GRK3 was localized to the nucleus accumbens, where GRK3 phosphorylation of the kappa opioid receptor was required for activation of p38 MAP kinase by the kappa-opioid receptor. Kappa opioid receptor activation also was required for increased immobility in response to forced swim stress [117]. Therefore, in nucleus accumbens activation of p38 MAP kinase was eliminated by GRK3 knock out. Because activation of kappa opioid receptors in nucleus accumbens causes aversive behavior that could contribute to the increased immobility after forced swim, this could explain the results in GRK3 knockout mice. However, GRK3 depletion has the opposite effect in other regions of the central nervous system. For example, spinal neuropathic pain, which requires kappa opioid receptor desensitization, is not observed in GRK3 knockout mice [80]. Therefore, GRK3 may differentially affect GPCR signaling depending on the regional localization. Hence, it is suggested that t in LC GRK3 participates in the termination of G protein mediated signaling by the α_{2A}-AR and CRF_1-R receptors.

5.1.3 Changes in GRK3, α_{2A}-AR and CRF_1 levels go in opposite direction with the disappearance of LH behavior

LH behavior induced by single bout of inescapable stress is reversible in nature. Therefore another aspect of the study was to assess what happens to

GRK3, α_{2A}-AR, and CRF_1-R levels when LH rats behave similarly to TC and NH rats i.e. when the escape deficits in LH rats disappear. Evident from the literature [23, 31, 87, 88] and results from this study, 120h post stress LH rats have similar escape latencies and escape failures compared to TC and NH animals. At 144 h post- stress (24 h after second shuttle box)**;** there is no longer any distinction in GRK3, α_{2A}-AR, and CRF_1-R levels between LH and NH animals in LC/amygdala, which goes well with escape task data, showing no behavioral distinction. Results indicate that 144 h post-stress there is an increase of GRK3 levels in LC/amygdala of LH and NH rats with corresponding decrease in α_{2A}-AR and CRF_1-R levels as compared to TC.

The time course of changes in GRK3 (**Fig 39)** in LC suggest that the biochemical distinctions starts at 1h post- stress wherein LH rats shows decreased GRK3 and corresponding increase in α_{2A}-AR and CRF_1-R levels . However 144h post -stress, GRK3 levels in LH animals show an increase similar to NH animals accompanied by down-regulation of α_{2A}-AR and CRF_1-R levels. These results are in the opposite direction to 48 h post-stress data wherein GRK3 was reduced in LH rats and no down-regulation of receptors. Biochemically 144h post-stress, stressed rats no longer fall into two distinct clusters, suggesting whatever changes that are causing LH rats to behave normally are occurring in NH animals too. However, the quantum of change is approximately 45% more in LH animals (GRK3, α_{2A}-AR and CRF_1-R levels from

post stress 1h to post stress-144h post-stress→70%, 78% and 59%, for LH vs. 33%, 13%, and 24% for NH rats). This reversion to normal behavior exhibited by LH animals can be attributed to their recovery and subsequent increase of GRK3 levels to that of NH animals which is enough to produce similar level of changes in α_{2A}-AR and CRF_1-R levels producing similar behavioral responses. LH and NH animals at 120h post-stress behave similarly to TC. However, it should be noted that NH and LH rats remain biochemically distinct from TC rats at this time, likely because LH and NH rats were initially stressed.

The LC is the primary noradrenergic nuclei in brainstem with NE as its major neurotransmitter. Tyrosine hydroxylase (TH) is the rate limiting enzyme involved in the synthesis of NE. It has been reported that during acute stress there is increased activation of noradrenergic neurons in LH rats and increased TH levels [118, 119]. Thus time dependent changes in this protein can be considered as an indirect estimation of neuronal function in LH and NH rats. LH animals start with 19% decrease 1h post-stress which goes up to 30% increase at 144h post-stress (49% change), however NH rats start with 20% increase 1h post-stress which goes down to 19% decrease at 144h post-stress (39% change) **(Fig 39)** . This also suggests that NH rats were different from LH rats starting from the beginning of exposure to stress. At 144h post-stress LH rats show increased and NH rats show decreased TH levels suggesting that

Locus Coeruleus

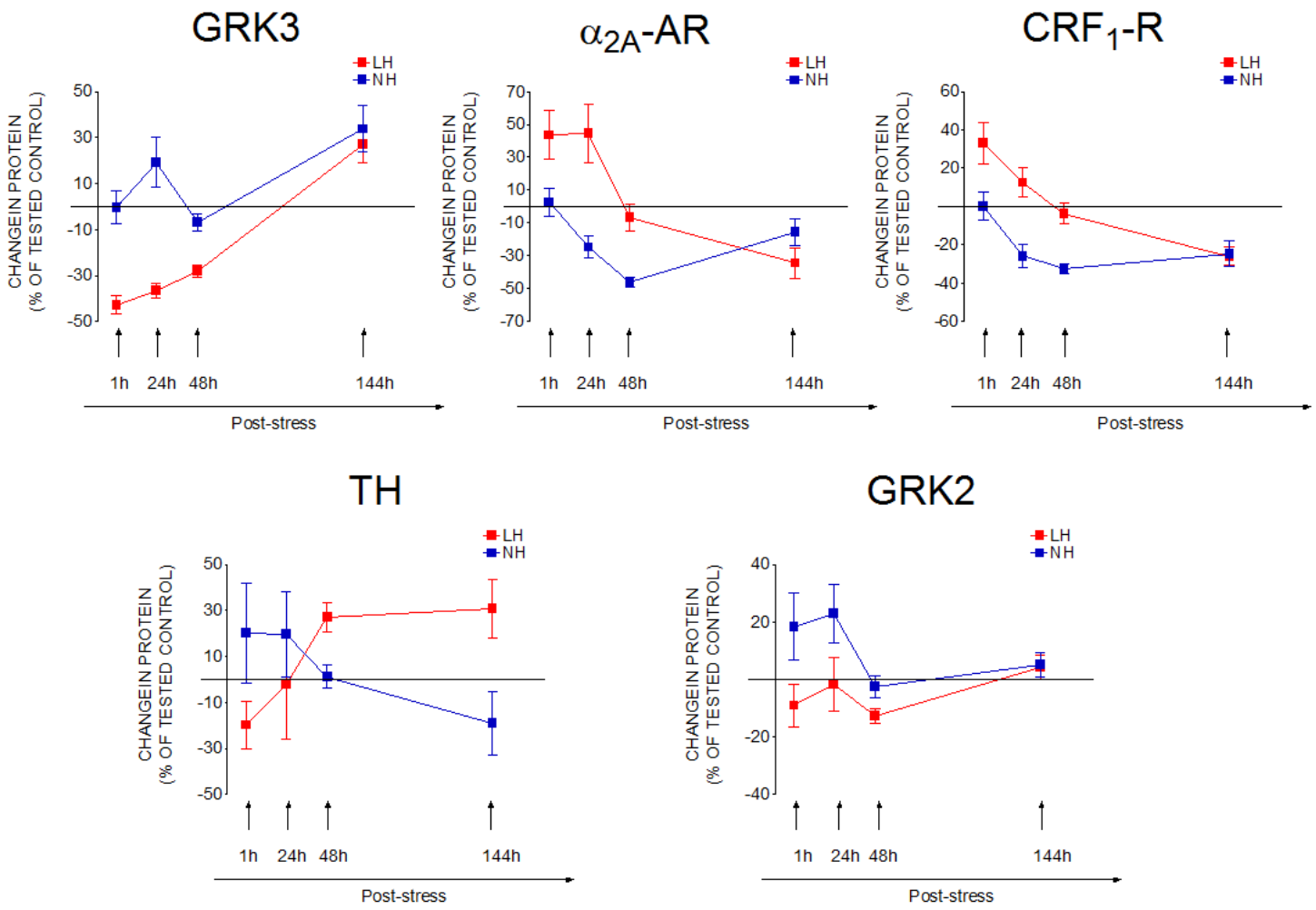


Figure 39: Time course of changes in protein in locus coeruleus: 1h-post-stress till disappearance of LH behavior (144h- post-stress)

Mean protein/loading control ratios of Learned helpless (LH) and Non-helpless (NH) rats expressed as % change with respect to mean protein/loading control ratios of tested control (TC) rats at respective time points (1h, 2 4h, 48h, and 144h- post-stress

compensation is occurring at different rates, thus allowing them to come to similar biochemical changes required to show similar behavior.

At 120h post-stress, there was no behavioral distinction within singly stressed rats. This suggests that the removal of stress was allowing rats to recover from stress and in addition, the biochemical machinery of the rat is trying to compensate for the losses due to stress exposure. The second shuttle box was done when rats have recovered from the previous exposure. LH and NH rats now being in the stress sensitization phase [23, 120, 121], considered even second shuttle box test stressful enough to cause compensatory biochemical changes. This might be one of the reasons for an increase in GRK3 levels in LH and NH rats, which allow them to down–regulate receptors and thus produce a behavioral response similar to TC rats.

The time course of biochemical events in amygdala (**Fig 40)** suggest that though there is no biochemical distinction into two groups at 1h post-stress , but 24h and 48h post-stress biochemical distinction occurs. Similar to LC, 144h post-stress GRK3 levels in the amygdala of LH animals show an increase similar to NH animals accompanied by down-regulation of α_{2A}-AR and CRF_1-R levels. Based on GRK3, α_{2A}-AR and CRF_1-R data, 144h post-stress rats no longer fall into two distinct clusters. However, the quantum of change is approximately 22% lower in LH rats (GRK3, α_{2A}-AR and CRF_1-R levels from 1h to post stress-144h post-stress →51%, 44% and 64%, for LH vs. 69%, 68%, and 88% for NH rats),

though rate of change in LH animals is lower in amygdala but it is not that drastic a quantum of change as it was seen with LC of LH animals.

In our study, cortex showed similar changes in GRK3, α_{2A}-AR and CRF_1-R levels in LH and NH rats at 24h, and 48h post-stress, suggesting that changes are stress dependent, rather than behavioral specific changes as occurred in LC and amygdala. At 144h post-stress, in cortex there are no biochemical changes (GRK3, α_{2A}-AR and CRF_1-R levels) in LH and NH rats suggesting effects of stress are no longer present **(Fig 41).**

Amygdala

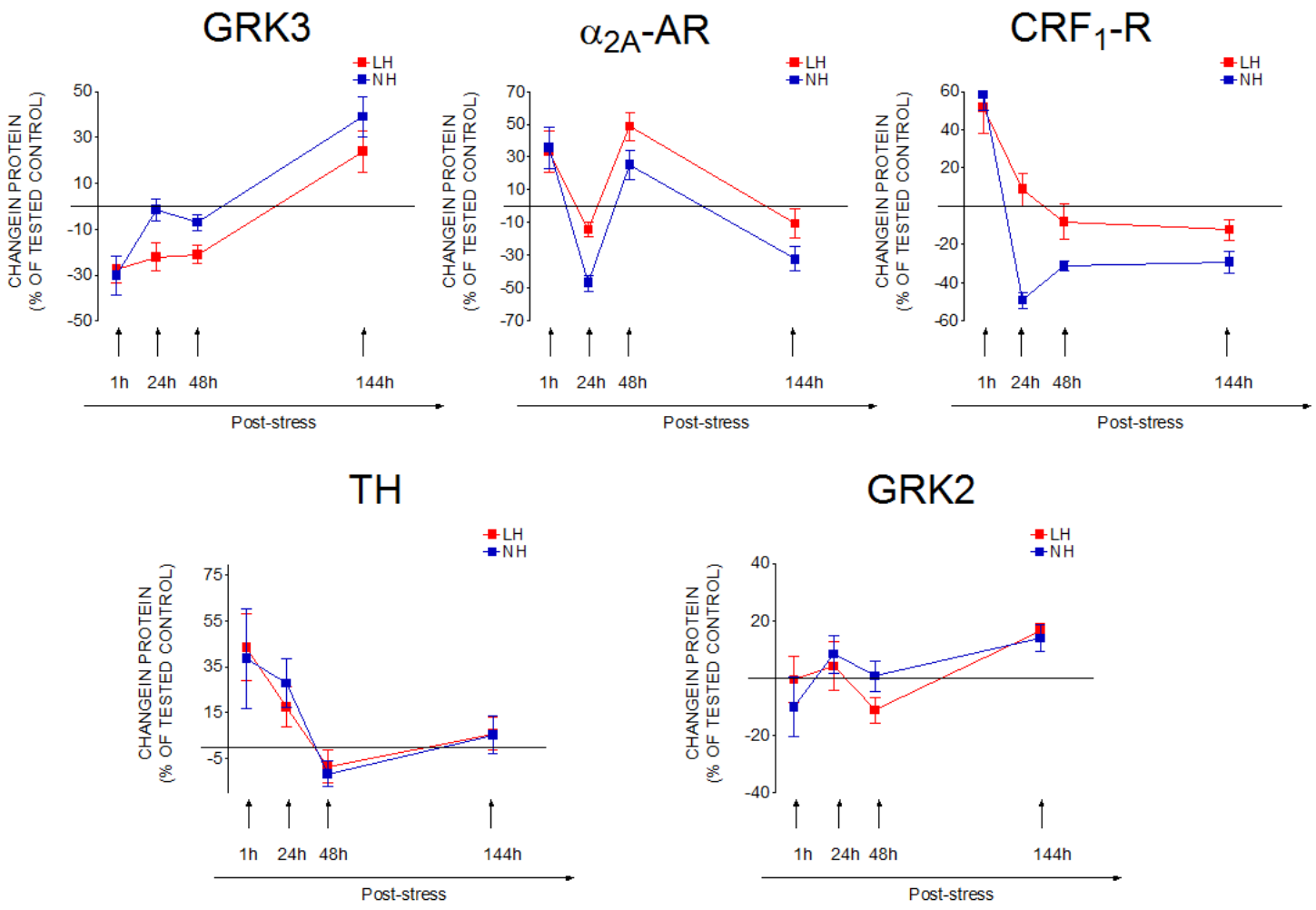

GRK3
LH
NH
CHANGEIN PROTEIN (% OF TESTED CONTROL)
50
30
10
-10
-30
-50
1h
24h
48h
144h
Post-stress
α2A-AR
LH
NH
CHANGEIN PROTEIN (% OF TESTED CONTROL)
70
50
30
10
-10
-30
-50
-70
1h
24h
48h
144h
Post-stress
CRF1-R
LH
NH
CHANGEIN PROTEIN (% OF TESTED CONTROL)
60
40
20
-20
-40
-60
1h
24h
48h
144h
Post-stress
TH
LH
NH
CHANGEIN PROTEIN (% OF TESTED CONTROL)
75
55
35
15
-5
1h
24h
48h
144h
Post-stress
GRK2
LH
NH
CHANGEIN PROTEIN (% OF TESTED CONTROL)
40
20
-20
-40
1h
24h
48h
144h
Post-stress

Figure 40: <u>Time course of changes in protein in amygdala: 1h-post-stress till disappearance of LH behavior (144h- post-stress)</u>

Mean protein/loading control ratios of Learned helpless (LH) and Non-helpless (NH) rats expressed as % change with respect to mean protein/loading control ratios of tested control (TC) rats at respective time points (1h, 24h, 48h, and 144h- post-stress)

Cortex

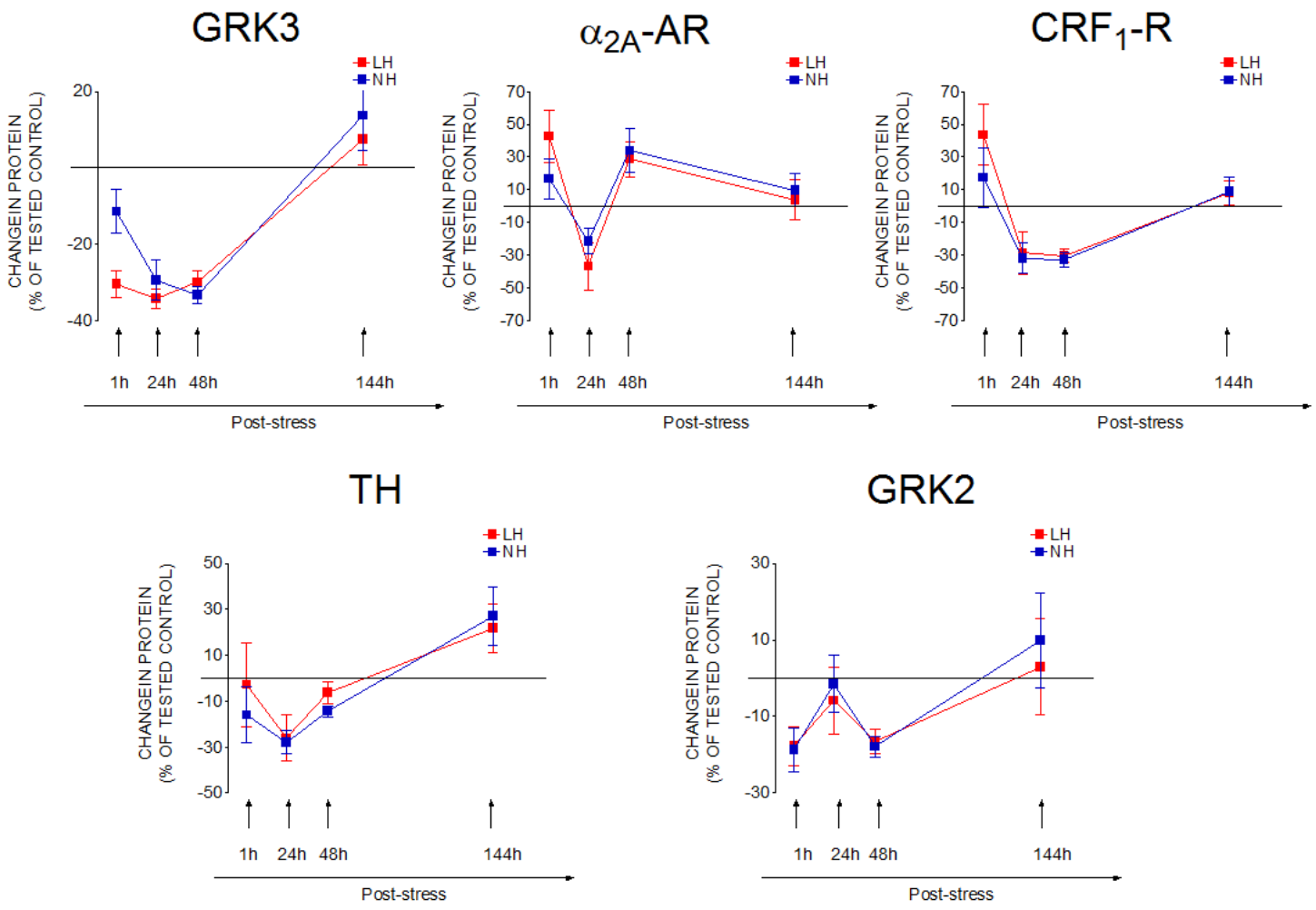

GRK3
α2A-AR
CRF1-R
TH
GRK2
LH
NH
CHANGEIN PROTEIN
(% OF TESTED CONTROL)
1h
24h
48h
144h
Post-stress

Figure 41: <u>Time course of changes in protein in cortex: 1h-post-stress till disappearance of LH behavior (144h- post-stress)</u>

Mean protein/loading control ratios of Learned helpless (LH) and Non-helpless (NH) rats expressed as % change with respect to mean protein/loading control ratios of tested control (TC) rats at respective time points (1h, 24h, 48h, and 144h- post-stress).

5.2 Repeated stress induces prolonged changes in behavior

Single stress induces readily reversible learned helpless (LH) behavior. However when LH rats show normal behavior, changes in GRK3, α_{2A}-AR and CRF_1-R levels among single stress rats do not suggest two population distinctions at 144h post-stress. Therefore there is a need to develop a validated model that can induce prolonged changes in behavior and it is very important from the viewpoint of answering questions pertaining to differences between readily reversible and not readily reversible LH behavior . Moreover what are the biochemical changes (GRK3, GRK2, TH, α_{2A}-AR and CRF_1-R levels) that lead to prolonged behavioral changes?

Taking the time course of reversal of LH behavior into consideration rats were exposed to tail shock stress sessions at a 3 day interval (Day 1, 4, 7) and a model of repeated stress-induced prolonged LH behavior lasting at least 14 days was developed. In this model among the stressed rats 50% LH and 50% NH rats were found. LH rats have significantly higher escape latencies and escape failures which were not readily reversible within 14 days (Day 8 (24h after last bout of TSS) to Day 22). In addition LH rats have significantly higher plasma corticosterone, epinephrine and CRF levels on day 23 post-stress (24h after last escape testing). This raises the interesting question of why changes in plasma level of these hormones were not observed in NH rats, as these animals also were repeatedly stressed. The concept of NH rats being stress resilient is

supported by this observation, as the results imply that the NH group is capable of overcoming the negative consequences of stress.

Studies show that when tail shock stress is given two times, LH behavior is prolonged for only 7 days after the second shock treatment [122, 123]. However the time window between the two stress exposures was 7 days, and escape behavior data from single stress show that the duration of LH behavior is 3 days only. Thus the reported study has shown the prolonged LH behavior (50% less than the repeated stress model used in this study; 7 vs. 14 days), and in between stress exposure, the rats have recovered from being LH. These data suggest that while LH behavior after a single stress bout disappears, there may be persistent increased susceptibility for development of a more prolonged LH behavior by second stress bout. Interestingly, this persistent risk outlasts the single stress-induced changes in GRK3, α_{2A}-AR and CRF_1-R levels in LC of NH vs. LH rats. However, stress-induced changes in both LH and NH rats relative to TC rats still exist and these may be contributing factor to this persistent risk.

Repeated exposure of stress also showed a 50% distributed bimodal population, which raises one more question of why even after three bouts of tail shock stress, the % of LH rats among stressed rats did not increase. If the population distribution of stressed rats is studied, it appears to be a continuum ranging from highly stress resilient to highly stress susceptible rats. The issue thus becomes, do 50% rats show LH behavior because single stress is only

sufficient, or, is it the difficulty of the escape task that differentiates the continuum of stressed rats into two halves (LH-stress susceptible, NH-stress resilient)? The repeated stress model suggests that it is the difficulty of escape task that is responsible for identifying 50% each of LH and NH populations among stressed rats. The reason is that increasing the dose of stress (3 bouts in repeated stress model) increases the % of escape failures of rats exhibiting LH behavior rather than increasing their proportion among repeatedly stressed rats **(Fig 42)**. It can be questioned why only increase in escape failures and no increase in escape latencies was observed in repeated stress-induced LH rats. This is due to the ceiling effect of the cutoff point of escape task (30s is the maximum limit), which means that even if repeated stress-induced LH rats have increased escape latencies, it would be masked by the arbitrary 30s maximum set for the completion of escape task (23.7s for single stress and 25.8 for repeated stress LH rats). Therefore, in order to increase the % of LH rats among stressed rats, the difficulty of the escape task needs to be increased as opposed to increasing the dose of stress (number of stress bouts).

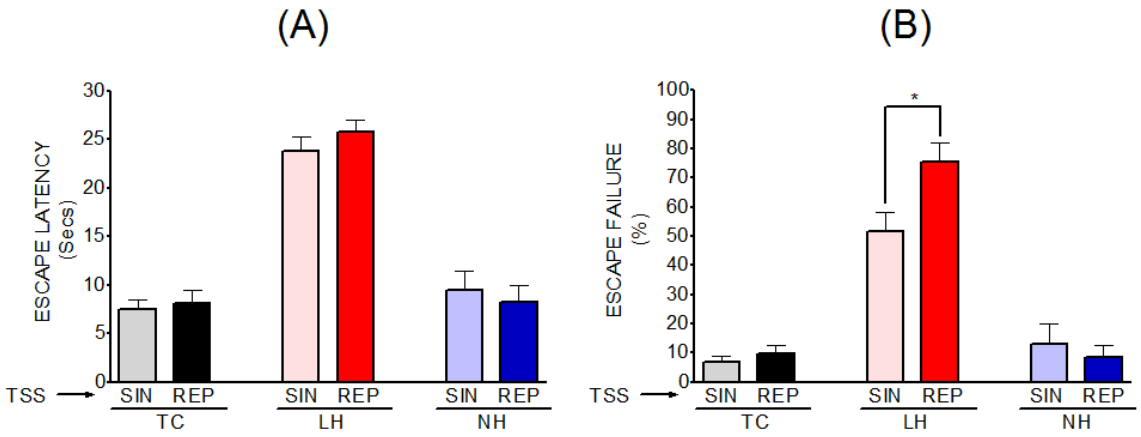


Figure 42: <u>Increased escape failures in single versus repeated stress-induced LH rats: Comparison of escape behavior data of single and repeated stressed rats</u>

Day 8 (24h after last bout of repeated stress protocol) of repeated stressed rats and 24 h post-stress of single stressed rats escape latencies and escape failures data was subjected to K-means cluster analysis, which identified the presence of two populations within stressed rats (LH-higher, NH-similar escape latencies/escape failures to TC).

Panel (A) Comparison of single and repeated stress-induced mean escape latency of TC, LH and NH rats, **Panel (B)** Comparison of single and repeated stress-induced mean escape failures of TC, LH and NH rats. Data is expressed as Mean ± SEM, and * - mean escape failure significantly different , LH (day 8 (repeated stress) vs. LH 24h (single stress) ($p \leq 0.05$) n=5-12.

SIN-single, REP-Repeated.

Results from other behavioral testing paradigms indicated that only the modified forced swim stress (MFSS), but not the open field test (OFT) and elevated plus maze (EPM), detected significant behavioral changes in LH vs. NH, TC and NC rats. There was a significant increase in immobility behavior and a significant decrease in swimming behavior in LH rats while NH rats behave similar to controls. When the 15 min results of MFSS were broken down in 5 min intervals, for first 5 minutes, there were no significant differences between LH and NH rats, however in last 10 minutes differences appear. These behavioral tasks also point towards the strong convergent and discriminant validity of the repeated stress model. Convergent validity is the level of similarity to which a model correlates with the outcomes of another model measuring the same construct. Discriminant validity is the level of dissimilarity to which a model correlates with the outcomes of another model measuring a different construct [3, 124].

OFT and EPM are tests done to measure anxiety and it has been seen that the single stress LH model which is used to screen antidepressants drugs, gave false positive screening results with anti-anxiety drugs (e.g. buspirone, an anti-anxiety drug prevents the development of LH behavior) [125]. Thus this repeated stress -induced prolonged LH behavioral model shows good discriminant validity as no increase in anxiety is detected in LH rats. However, MFSS showed distinction in behaviors among LH and NH rats. MFSS is another

model used to screen antidepressant drugs. Thus similar discrimination into behaviors achieved by models measuring same construct of depression associated behavior suggest that repeated stress model has sound convergent validity. Another explanation could be that MFSS is more stressful than OFT and EPM and it is exposure of rats to a particular intensity of stress which governs behavioral distinction of LH and NH rats. The previous prolonged LH behavioral model reported by Dwivedi and his colleagues [122, 123]; did not test their models on the above said validities. For this reason, the repeated stress model reported here is more relevant to stress- induced prolonged behavioral changes.

5.3 Repeated stress induces behavioral specific changes in GRK3, GRK2, α_{2A}-AR and CRF_1 levels

The repeated stress-induced prolonged LH behavioral model was created for answering two questions a) Are there any changes at the level of GRK3, α_{2A}-AR and CRF_1 in the repeated stress model of LH and b) Are the changes cumulative resulting in prolonged LH behavior different from those observed with single stress-induced LH behavior?

Results indicated that reduced levels of GRK3 in LC is associated with prolonged learned helpless behavior amongst a population of rats subjected to repeated stress, whereas NH rats exhibit normal GRK3 levels in this brain area. Moreover, reduced levels of GRK3 are associated with up- regulation of α_2-AR

and CRF_1-R in LC of repeated stress LH rats. Quantitatively, GRK3 was reduced to similar levels in single and repeated stress LH rats. Therefore why does repeated stress exposure prolong LH behavior?

The point of difference between single and repeated stress was receptor levels, α_{2A}-AR and CRF_1-R. In the single stress condition, NH rats showed down regulation of α_{2A}-AR and CRF_1-R which allowed them to adapt to stress but LH rats having lower GRK3 levels were unable to down-regulate α_{2A}-AR and CRF_1-R. In contrast, with the repeated stress condition, NH rats did not show any down regulation, but LH rats showed up-regulation of α_{2A}-AR and CRF_1-R. On a quantitative scale LH rats in both single and repeated stress conditions have higher level of α_{2A}-AR and CRF_1-R pointing to defective down-regulation of receptors. These differences in LH rats in single and repeated stress can be due to differential responses to two types of stresses. Single stress caused needed down regulation of receptors in NH rats to maintain their normal behavior, while in repeated stress, the biological system has compensated to a steady state, where receptor levels in NH rats should be at basal level to maintain behavior similar to control. In both conditions of stress LH rats showed defective down regulation due to reduced GRK3 levels.

In contrast to GRK3, GRK2 was significantly reduced in LH rats after repeated stress but not after single stress. This might suggest that effects of repeated stress might have cumulative effect on reduction of the GRK2/3 family.

In addition, during single stress, TH levels were increased in LH rats, but they were decreased after repeated stress again suggesting effects of repeated stress are different from single stress. These differences are well supported in the literature, wherein acute stress results in increased activation of noradrenergic neurons in LH rats and increased TH levels [118, 119]. However repeated exposure of stress can be so intense that it may reduce LC tissue NE [91] and which can be indirectly correlated to decreased TH levels.

Similar to LC, amygdala of LH rats (repeated stress) showed similar reduction in GRK3 levels with increased α_{2A}-AR levels but no change in CRF_1-R, GRK2 and TH levels. The changes in α_{2A}-AR show relatively higher up-regulation in amygdala of LH rats under both single and repeated stress conditions. In the single stress condition, CRF_1-R levels were decreased in NH rats, but they were not changed in any of LH and NH rats after repeated stress, again suggesting effects of single and repeated stress are different. These results suggest that effects in LC are more prominent and changes in amygdala are associated with changes in LC by its neurophysiological interaction with amygdala.

Repeated stress did not show any biochemical changes in cortex of LH and NH rats. This implies that whatever happens in these two groups, both show similar patterns in cortex, as was seen in single stress condition wherein GRK3 was reduced in both LH and NH rats along with similar level of changes in α_{2A}-AR and CRF_1 levels. The fact that repeated stress did not generate any changes

in cortex further suggests that the effects of stress are no longer present and the behavioral consequences are result of changes in LC followed by amygdala.

Repeated stress-induced changes in LC at the level of GRK3, α_{2A}-AR and CRF_1-R levels but not in amygdala and cortex can be used to independently identify stress resilient NH and non-resilient LH populations within a group of stressed rats. Cluster analysis of GRK3, α_{2A}-AR and CRF_1-R levels of repeatedly stressed rats in cortex and amygdala showed no cluster formation. Cluster analysis using levels of GRK3, α_{2A}-AR and CRF_1-R as three simultaneous variables suggests that LC showed 94% agreement with the behavioral data (**Fig 43)**. In addition since there were reductions in GRK2 levels, cluster analysis using levels of GRK2 alone or simultaneous with α_{2A}-AR and CRF_1-R levels was done. In both cases there was only 50% agreement with the behavioral data. Therefore it is suggested that the changes in GRK3, α_{2A}-AR and CRF_1-R in LC are most closely associated with the prolonged LH or NH behavior after repeated stress and changes in amygdala are just consequences of changes in LC. This is different from single stress results as amygdala though not as accurate as LC in predicting stress resilience and stress susceptibility showed some independent association with LH behavior.

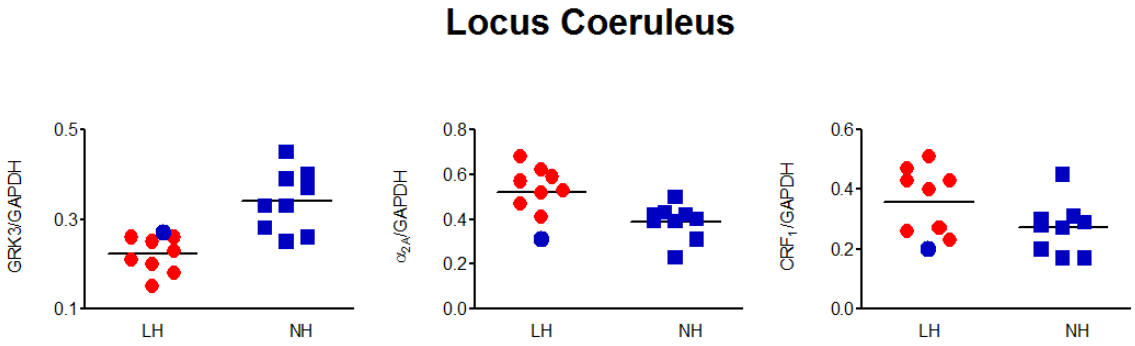


Figure 43: <u>Comparison of population with in the repeated stressed group of rats identified by cluster analysis of escape behavior data versus protein levels</u>.
In each panel, circles represent data points from LH rats while squares represent data points from NH rats as determined by behavioral testing. Blue and red symbols represent the two populations identified on the basis of the protein parameter represented in each panel.

Finally, changes at the level of GRK3, accompanying impaired regulation of α_{2A}-AR and CRF_1-Rs in LC is compounded by reductions in GRK2 and TH levels which might have role to contribute towards maintenance of LH behavior.

The LH paradigm is used mainly for screening antidepressants drugs and α_{2A}-AR/CRF_1-R are well studied targets for antidepressants. Up-regulation of α_{2A}-AR in LC of patient's suffering from major depression further support the idea of impaired regulation of this receptor due to decreased GRK3 in rats exhibiting prolonged LH behavior [83, 126, 127]. In addition, the observation that CRF_1-R antagonists prevent the development of LH behavior [90]; support the concept of increased activity of CRF on CRF_1-R which is also supported by our observation of increased CRF_1-Rs in the LC of repeated stress LH rats. Thus changes in levels of both receptors are well correlated with reduction of GRK3 in LC.

Various reports now have shown the potential role of GRK3 in neuropsychiatric illnesses like bipolar depression and major depressive disorder. These reports suggest that GRK3 is reduced in brain tissues of patients suffering from bipolar depression [20]. In addition a subset of bipolar patients has a SNP (-382 G/A) in the promoter region of GRK3 which is associated with lower levels of GRK3 and higher propensity to develop stress initiated relapses of the disease [17-19, 81, 82]. Further, it was reported that not only levels of GRK3 changed, but GRK2 levels in platelets/mononuclear leucocytes were significantly reduced in major depressive disorder patients [21, 83, 128]. Considering what we see in a

rats exhibiting prolonged LH behavior, the associated changes in both GRKs are compatible with available literature of both GRKs being reduced in stress associated chronic affective disorders.

5.4 Reversal of the prolonged LH behavior by desipramine treatment restores GRK3, GRK2, and CRF_1 levels to normal and reduces α_{2A}-AR levels

Most of the available antidepressants (Tricyclic antidepressants (TCAs) and Selective Serotonin reuptake inhibitors (SSRIs) mainly) can prevent and/or reverse single stress-induced LH behavior. The question was, will these agents be effective in the repeated stress-induced LH paradigm. Clinical studies had shown that TCAs are more effective than SSRIs in patients with resistant and severe depression [129-133] . Secondly among the TCAs it has been shown that desipramine treatment reduces LC neuronal activity [134]. In addition our initial results in LC from single and repeated stress suggested impaired regulation of α_{2A}-AR/CRF_1 receptors. Therefore the catecholaminergic system was chosen as the first target in an attempt to reverse prolonged LH behavior. Fourteen days LH behavior in rats approximately equals 1.5 years of clinical depression in humans, hence desipramine, a TCA that is more effective in cases of prolonged and severe depression and targets the catecholaminergic system was selected [129-133, 135, 136].

Fourteen days of desipramine (5mg/Kg bid) treatment eliminated the increased escape latencies and escape failures of repeatedly stressed LH rats. They behaved similar to NH, TC and NC rats. In the repeated stress model, 3 bouts of stress segregated stressed rats into 50% NH and LH rats on day 8 (24h after last bout of stress). Escape latencies and escape failures on day 22 (14 days) after drug treatment were significantly reduced in LH rats as compared to day 8 showing escape behavior similar to control (NC or TC) and NH rats. Desipramine treatment also normalized the levels of epinephrine, CRF and corticosterone levels in LH rats. These results suggest that the repeated stress model not only exhibits behavioral and hormonal changes associated with stress-induced pathophysiology but shows reversal of these changes with clinical effective antidepressant treatment. Studies with the model described by Dwivedi and colleagues [122, 123] did not look at these validation parameters.

Reversal of prolonged LH behavior was accompanied by restoration of GRK3/2 levels in LC and adaptive down regulation of α_{2A}-AR (significant) and CRF_1-R (non-significant). This suggests that α_{2A}-ARs which were up-regulated in LH rats due to reduced GRK3 show an adaptive decrease after restoration of GRK3 levels. However a direct effect of desipramine as an SNRI causing increased synaptic NE concentrations and consequent down-regulation of α_{2A}-AR cannot be neglected in a noradrenergic abundant region. Similar to the changes in α_2-AR, CRF_1-R levels also were down-regulated by desipramine

treatment in LH rats, but these were not significant. One possible reason for greater effect on α_{2A}-AR might be a synergistic effect via restoration of GRK3 combined with higher synaptic NE concentrations.

Desipramine treatment also restored GRK3 levels in amygdala along with normalization of up-regulation of α_{2A}-AR similar to LC. CRF_1-R levels did not change before and after the desipramine treatment in amygdala. This was different from changes in LC wherein CRF_1-R was up-regulated before treatment and down-regulated after treatment. The idea of a combined effect of GRK3 restoration and increased NE concentration by SNRI in down-regulating α_{2A}-AR might be more relevant in LC, because direct effects of an SNRI are more prominent in a region of noradrenergic abundance in contrast to the amygdala. Cortex did not show any changes in GRK3/2, α_{2A}-AR, CRF_1-R, and TH levels before and after desipramine treatment in repeated stress-induced LH rats.

Results of the repeated stress paradigm have indirect support from studies in patients suffering from depression and taking chronic antidepressants drugs. Patients who respond to antidepressant drugs show restoration of GRK2/3 levels along with down-regulation of α_{2A}-AR [21, 83, 126, 127]. Although it is still embryonic to suggest any cause and effect relationship between GRK3 and LH behavior (for both single and repeated stress), it cannot be denied that levels of GRK3 along with changes in α_{2A}-AR, CRF_1-R are well correlated with behavioral

identification of stress resilient and stress susceptible populations in single and repeated stress studies.

In the present study it has been shown that there are spatial and temporal correlates of protein GRK3, α_{2A}-AR, CRF_1-R which can track with single stress-induced development and disappearance of LH behavior. These correlates extend to the new model of repeated stress-induced prolonged LH behavior. In summary, the present study suggests that reduced levels of GRK3 in LC after stress (both single and repeated) associate with stress susceptibility and contribute to the development and sustenance of LH behavior. Stress susceptibility also is associated with alterations of α_2-AR and CRF_1-R regulation in the LC after stress. Future studies will be directed towards determining if changes in GRK3 levels in LC are indeed a cause for development of learned helplessness and whether interventions that enable GRK3 levels in LC to be maintained may reduce or prevent the development of LH.

5.5 Perspectives

Until now a majority of antidepressant drugs that have come to the market have been tested for their efficacy in the single stress-induced LH paradigm. This model has the disadvantage of showing false positive results with drugs which are not used to treat depression (anti-anxiety drugs are also beneficial in these model). Repeated stress-induced prolonged LH behavior not only provides an

alternative to test chronic antidepressant treatments , but also to examine questions regarding why antidepressants take 3-4 weeks of treatment to show any clinical improvement in humans. The results of the present study also suggest that studies to understand why NH rats fail to develop escape deficits may be more important than LH itself.

The big question is why the single and repeated effect of any manipulation (in this study-stress) is different. It can be postulated that it is the body's adapting mechanism that governs the response, rather than manipulation producing any responses of its own. The answer lies in the survival/adaptable behaviors which we have gathered as a species during centuries by Darwinian selection advantage. However, the cost of these evolutionary benefits may be paid by increased vulnerability towards the predisposition of compensatory diseases [4, 137] .

In a process of selection for fighting starvation, evolution devised energy storing behavior leading to abdominal obesity and metabolic disorder predisposition. Similarly for fighting dehydration, evolution devised fluid and water saving behavior leading to predisposition to hypertension. In combat with adversaries, we developed fear and arousal behaviors and selection advantages predisposed us to anxiety and insomnia disorders. In the face of a dangerous situation, we tackled it by a way of social isolation and it predisposed us to depression like behavior. In combating injuries, we developed potent immune

responses, which gave us autoimmune disorders. In case of tissue injury, we selected retaining tissue integrity which leads us to predisposition to chronic fatigue syndrome. It is true that whatever we give it comes back and now at this phase of developed society we are getting all things back from the Darwinian selection criteria [4, 137].

6. SUMMARY AND CONCLUSIONS

1 The present study suggests that reduced levels of GRK3 in Locus Coeruleus (LC) after single exposure of tail shock stress is associated with stress susceptibility and contribute to the development and maintenance of learned helpless (LH) behavior. Stress susceptibility also is associated with impaired alpha$_{2A}$-adrenoceptors (α_{2A}-AR), and corticotrophin releasing factor 1 receptor (CRF_1-R) regulation in the LC after single and repeated stress.

2 The changes in GRK3, α_{2A}-AR, and CRF_1-R levels in LC of LH and non-helpless (NH) rats (single stress) starts as early as 1h post-stress and persist with the LH behavior. When LH behavior disappears, the changes in GRK3, α_{2A}-AR, and CRF_1-R levels between LH and NH rats are no longer distinct, but they still are globally different from control.

3 Similar to LC, amygdala also shows the similar pattern of reduced levels of GRK3 with impaired α_{2A}-AR, and CRF_1-R regulation. These changes are associated with single stress-induced LH behavior. However the biochemical distinction based on changes in GRK3, α_{2A}-AR, and CRF_1-R levels does not start as early as 1h post-stress.

4 Single stress exposure does not translate into prolonged behavioral changes. However, in present study a validated model of prolonged behavioral

changes was created wherein 3 bouts of tail shock stress (day 1, 4, and 7) generated prolonged LH behavior for at least 14 days.

5 Repeated stress shows reduction of GRK3 with relatively higher α_{2A}-AR and CRF_1-R in LC of LH rats. An additional reduction of GRK2 in these LH rats might contribute to maintenance of LH behavior. Amygdala also showed reduction of GRK3 with impaired α_{2A}-AR regulation in these LH rats.

6. Reversal of prolonged LH behavior by desipramine treatment was accompanied by restoration of GRK3/2 levels in LC with adaptive down regulation of α_{2A}-AR and CRF_1-R. Desipramine treatment also restored GRK3 levels in amygdala along with normalization of α_{2A}-AR levels. Repeated stress LH rats showed α_{2A}-AR up regulation in LC and amygdala; however after desipramine treatment the α_{2A}-AR levels in LC were down-regulated but normalized in amygdala of LH rats. This might happen due to the combined effect of GRK3 restoration and increased NE concentration by selective NE reuptake inhibitor (SNRI) in down-regulating α_{2A}-AR in LC, because direct effects of an SNRI are more prominent in a region of noradrenergic abundance in contrast to the amygdala.

7 In both conditions of stress (single and repeated) levels of GRK3 ,α_{2A}-AR, and CRF_1-R collectively in LC can predict the behavior of rat, independent of behavioral testing, suggesting levels of GRK3 ,α_{2A}-AR, and CRF_1-R in LC are well correlated with behavior of stressed rats.

7.REFERENCES

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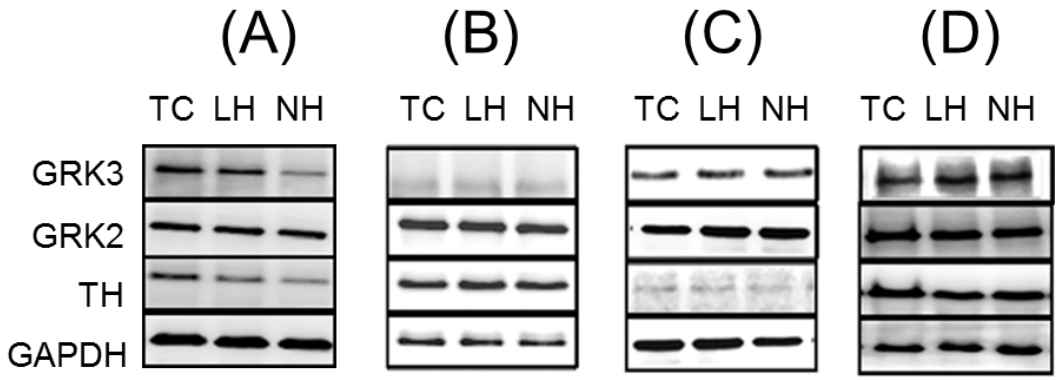
8.APPENDIX


(A)
(B)
(C)
(D)
TC LH NH
TC LH NH
TC LH NH
TC LH NH
GRK3
GRK2
TH
GAPDH

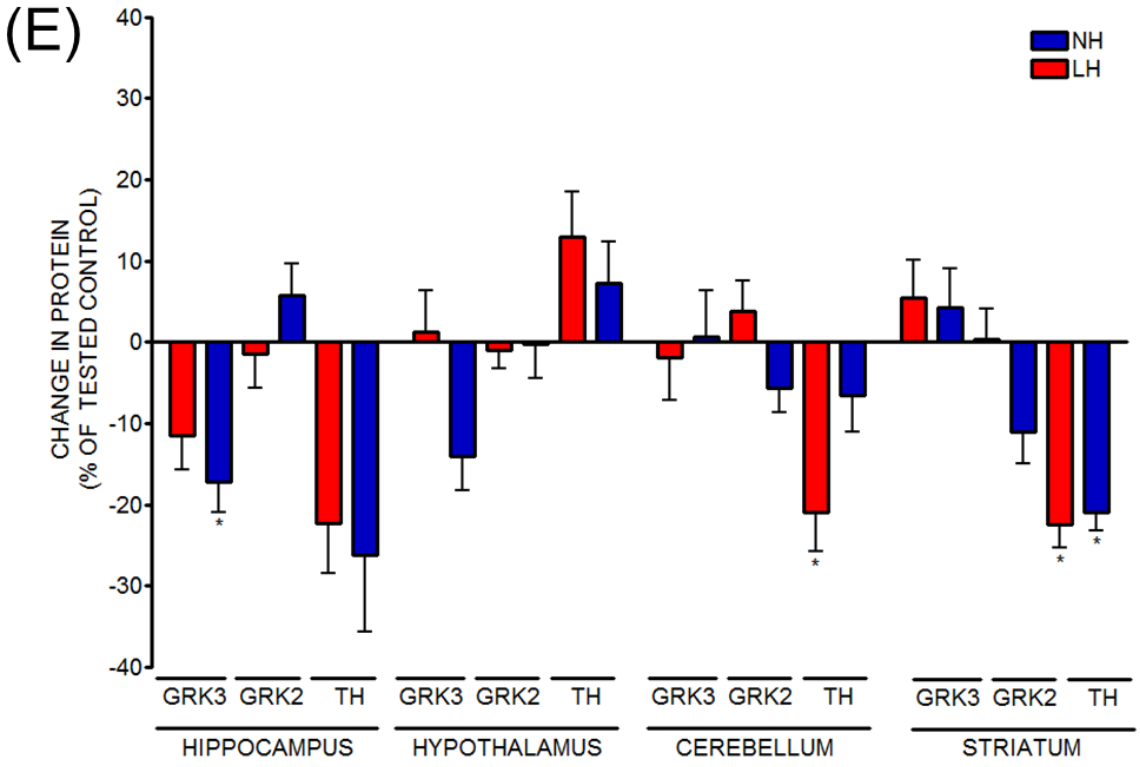

(E)
NH
LH
CHANGE IN PROTEIN (% OF TESTED CONTROL)
40
30
20
10
0
-10
-20
-30
-40
GRK3 GRK2 TH
GRK3 GRK2 TH
GRK3 GRK2 TH
GRK3 GRK2 TH
HIPPOCAMPUS
HYPOTHALAMUS
CEREBELLUM
STRIATUM

Figure A1: <u>Changes in GRK3, GRK2, and TH levels in hippocampus, hypothalamus, cerebellum, and striatum 48h post-stress (single stress)</u>
Tested control (TC) and stressed rats, 48h post-stress (24h after escape testing), were euthanized and their brains were removed. Hippocampus, hypothalamus, cerebellum, and striatum was isolated from brains of TC, LH and NH rats and levels of GRK3, GRK2, TH , α_{2A}-AR and CRF_1-R were measured using western blot technique **(For details refer to Fig 9 legend)**

Panel (A, B, C, D) Representative western blots for GRK3, GRK2, TH, and loading controls. **Panel (E)** Change in protein for LH and NH rats expressed as % of tested control. Significant change in protein levels ($p \leq 0.05$) *- (LH, NH vs.TC) n=8-9.

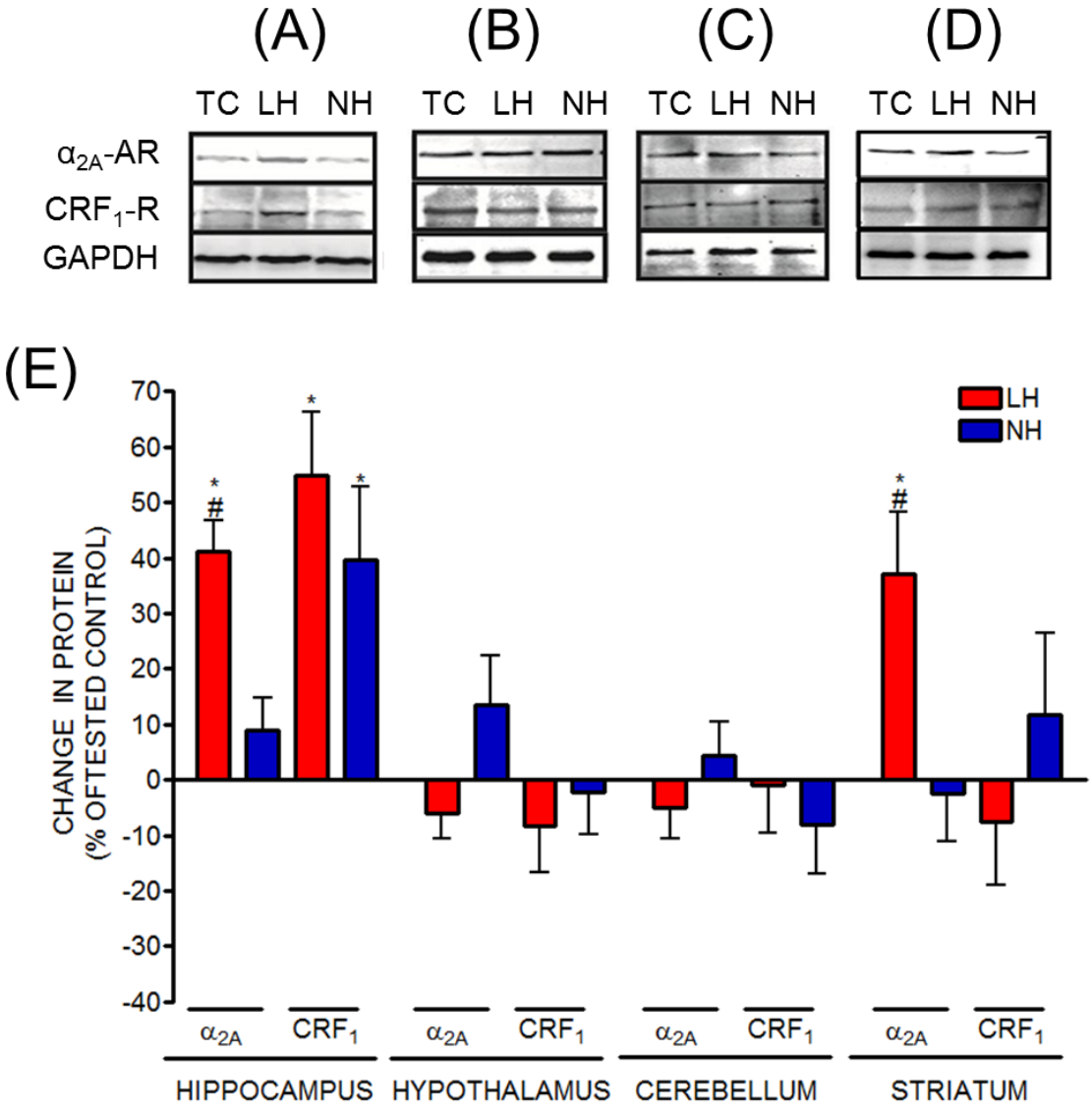

(A)
(B)
(C)
(D)
TC LH NH
TC LH NH
TC LH NH
TC LH NH
α2A-AR
CRF1-R
GAPDH
(E)
LH
NH
CHANGE IN PROTEIN
(% OFTESTED CONTROL)
70
60
50
40
30
20
10
0
-10
-20
-30
-40
α2A
CRF1
α2A
CRF1
α2A
CRF1
α2A
CRF1
HIPPOCAMPUS
HYPOTHALAMUS
CEREBELLUM
STRIATUM

Figure A2: Changes in α_{2A}-AR and CRF_1-R levels in hippocampus, hypothalamus, cerebellum, and striatum 48h post-stress (single stress)
Tested control (TC) and stressed rats, 48h post-stress (24h after escape testing), were euthanized and their brains were removed. Hippocampus, hypothalamus, cerebellum, and striatum was isolated from brains of TC, LH and NH rats and levels of α_{2A}-AR and CRF_1-R levels were measured using western blot technique **(For details refer to Fig 9 legend)**

Panel (A, B, C, D) Representative western blots for α_{2A}-AR, CRF_1-R, and loading controls. **Panel (E)** Change in protein for LH and NH rats expressed as % of tested control. Significant change in protein levels ($p \leq 0.05$) *- (LH, NH vs.TC) # - (LH vs. NH) n=7-9.