Changes in GRK3 and Norepinephrine Responsiveness in Locus Coeruleus Neurons are Associated with Learned Helplessness After Repeated Forced Swim Stress

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A Dissertation Presented to

The Department of Pharmacological and Pharmaceutical Sciences

University of Houston

In Partial Fulfillment of

the Requirements for the

Doctor of Philosophy Degree

By

Kaustuv Saha

August 2011

Dedicated to my family …

My Father

My Mother

My Brother

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ABSTRACT

In brain, locus coeruleus plays an important role in mediating the stress responses. Two important neurotransmitter/hormones released in locus coeruleus (LC) during exposure to stress include corticotrophin releasing factor (CRF) and norepinephrine (NE). CRF and NE predominantly act on G protein-coupled receptors (GPCR), the corticotrophin-releasing factor type 1 receptor (CRF_1-R) and the alpha$_{2A}$–adrenoceptors (α_{2A}-AR), respectively. Activation of CRF_1-R increases LC neuronal firing and activation of postsynaptic α_{2A}-AR inhibits firing of LC neurons. Additionally, presynaptic α_{2A}-ARs act as autoreceptors inhibiting release of NE in LC. Both the agonist-occupied CRF_1-R and α_{2A}-AR are preferentially desensitized by G protein-coupled receptor kinase 3 (GRK3). This desensitization contributes to termination of the signaling of CRF_1-R and α_{2A}-AR, observed during the persistent presence of neurotransmitters during stress. At present, there is a gap in knowledge as to what changes in GPCR signaling occur during single and repeated stress. The present study provides a contribution towards filling this gap. This study observed the effects of single and repeated forced swim stress on the escape task performance of rats in a shuttle-box. The study also observed the effects of GRK3 and NE responsiveness in LC neurons in slices of rat brainstem. Both

single and repeated forced swim stress segregated the stressed rats into two clusters based on their performance in the escape task. One cluster showed impaired escape behavior compared to controls and was designated Learned Helpless (LH), showing susceptibility to the adverse effects of stress. The other cluster of stressed rats showed escape behavior similar to the controls and was termed Non-Helpless (NH), showing resilience against the adverse consequences of stress. Thus this study demonstrated that a milder stress than inescapable electric shock, the stress paradigm of forced swim, could induce deficits in escape behavior. These deficits are a well-established index of depression-associated behavior. Biochemical analysis showed that single forced swim stress did not cause any change in the levels of GRK3, CRF_1-R and α_{2A}-AR in the LC of the LH rats compared to the control and NH rats. However, repeated forced swim stress caused a decrease in the levels of GRK3 and an increase in the levels of CRF_1-R and α_{2A}-AR in the LC of LH rats compared to the control and NH rats. Also, repeated forced swim stress was accompanied by an increase in the responsiveness of α_{2A}-AR upon application of lower concentrations of NE as observed by measuring the changes in membrane current in response to different concentrations of NE in single LC neuron. Moreover, the increases in immobility, LH behavior, decreases in GRK3 and increase in CRF_1-R and α_{2A}-AR in LC after repeated forced swim stress alone were not observed in rats pretreated with desipramine (DMI). In conclusion, a

much milder and more physiological stressor than electric shock, repeated forced swim stress, enables the identification of a sub-population of stress-susceptible rats that display LH. This LH behavior was associated with a decrease in GRK3 and an increase in α_{2A}-AR levels and responsiveness in LC, accompanied by an increase in CRF_1-R levels. The repeated forced swim-induced changes in responsiveness of the postsynaptic α_{2A}-AR may indicate that when the stressful stimuli are removed there is a rebound compensatory mechanism by which the α_{2A}-AR may reduce LC hyperactivity. Although, the method used in this study measured only postsynaptic α_{2A}-AR function, if similar changes in the presynaptic α_{2A}-AR occur, this would decrease NE release in LC during stress. Thus the pre- and postsynaptic α_{2A}-ARs would cancel each other functionally. This would lead to predominance of excitatory effects of CRF_1-R, potentially contributing to the hyperresponsiveness of LC to CRF and to hyperactivity of the LC that is characteristically observed on exposure to stress. DMI pretreatment, by increasing the availability of norepinephrine in the LC, will maintain an inhibitory tone on the neurons and prevent the hyperactivity of LC neurons associated with exposure to repeated stress. This will lead to prevention of repeated forced swim stress-induced decrease in GRK3 and increase in the levels and responsiveness α_{2A}-AR and CRF_1-Rs in the LC, resulting in the prevention of subsequent impairment of escape behavior in rats.

CONTENTS

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

DMI – Desipramine

DNC – Desipramine treated non-swim control

DRSS – Desipramine treated repeated sham swim control

DRFS – Desipramine treated repeated forced swim stress

EDTA - Ethylene Diamine Tetra Acetate

EPM - Elevated Plus Maze

GPCR - G Protein-Coupled Receptor

GRK - G Protein-Coupled Receptor Kinase

GRK2 - G Protein-Coupled Receptor Kinase -2

GRK3 - G Protein-Coupled Receptor -3

HSP - Heat Shock Protein

LC - Locus Coeruleus

LH - Learned Helpless

Na_2EDTA - Sodium Ethylene Diamine Tetra Acetate

Na_2SO_3 – Sodium Sulfite

NC - Non-swim 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

PVDF – Polyvinyldifluoride

RFS – Repeated Forced swim stress

RSS – Repeated sham swim control

SDS - Sodium Dodecyl Sulfate

SEM - Standard Error of Mean

SFS – Single forced swim stress

SNP - Single Nucleotide Polymorphism

SSS – Single Sham swim control

TCA -Tricyclic antidepressant

TH – Tyrosine Hydroxylase

α_{2A}-AR - Alpha$_{2A}$-Adrenoceptor

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1. INTRODUCTION

One in every four adult individuals (approximately 26.2%) in the United States is diagnosed with symptoms of one or more mental disorders and 22.3% of these cases can be categorized as severe (1-3). Some of these ailments constitute mood disorders such as major depressive disorders (US adult prevalence of 9.5%), personality disorders (US adult prevalence of 9.1%) and anxiety disorders (US adult prevalence of 18.2%) (2). The annual total direct and indirect costs related to serious mental illness has been estimated by the National Institute of Mental Health (NIMH) to be above $300 billion (4). Moreover, the Centers for Disease Control and Prevention have projected depression to be the second most important cause of disability worldwide by 2020, second only to ischemic heart disease (5).

Stress reportedly contributes to the development and maintenance of many psychiatric disorders (6). Evidence implicates “psychosocial” stress to be associated with depression and anxiety (7-9). Stress might be described as a noxious stimuli that threatens the psychological and physiological homeostasis of a living organism (10). The exposure to the adverse stressors leads to a chain of coordinated biological reactions referred to as the “stress responses” (10, 11). The stress responses constitute an intricate, coordinated and profoundly

conserved phenomenon involving the central nervous and endocrine systems, devised by nature to brace the organism to respond physiologically and behaviorally in order to survive the challenge (10). The stress responses activate adaptive mechanisms, maintained as long as the stress exposure is there and terminated eventually (recovery) when the stress is abolished. This active and dynamic process of adaptive responses involves brain, endocrine (which incorporates hypothalamic-pituitary-adrenal axis stimulation), autonomic (incorporating cardiovascular, gastric and intestinal activities), immunological, and behavioral and cognitive (hyperarousal and changes in attentive behavior) components. The purpose of this process is to preserve homeostasis and the process is collectively referred to as “allostasis” (10).

A conglomerate of biological mediators (including cortisol, monoamines, cytokines, opioids, corticotrophin releasing hormone) acts to preserve physiological viability through the process of allostasis (12). These mediators function in an interactive manner by influencing the levels and function of each other in a spatio-temporal pattern (13). However, upon exposure to repeated or chronic stress, there can be malfunctional allostasis or the improper termination of allostasis, leading to adverse consequences in the body, a phenomenon termed “allostatic load” (13).

The brain performs a central role of coordinating all the changes that occur from the perception of the stimuli to the orchestration of the responses

during stress. Amongst the various brain regions associated with or influenced by the stress responses, the important ones are prefrontal cortex (associated with executive functions and memory relevant to current happenings and planning), hippocampus (responsible for “episodic and declarative” memory and circumstantial memory), amygdala (associated with fear, anxiety and labeling the memory with emotion), nucleus accumbens, hypothalamus (activation of hypothalamic-pituitary-adrenal axis and control of the autonomic nervous system) and locus coeruleus (major noradrenergic source in the brain, associated with arousal) (14, 15).

Both in man and in animals the limbic-hypothalamic-pituitary-adrenocortical (LHPA) axis and the central and peripheral noradrenergic systems are stimulated during stress and are thought to play a vital role in eliciting the stress response (16-20). There are reports of extensive interactions between two important stress mediator systems, namely corticotrophin-releasing factor (a component of hypothalamic-pituitary-adrenocortical axis) and norepinephrine, in the brain (21). The main norepinephrine (NE) source in brain, the locus coeruleus (LC), projects to diverse regions like amygdala and hypothalamus as well as cortex, hippocampus and dorsal raphe. On the other hand, the LC receives innervations from various brain regions like the central nucleus of the amygdala, paraventricular nucleus of hypothalamus, nucleus tractus solitarius, nucleus prepositus hypoglossi and dorsal raphe nucleus, each with diverse

functions associated with the stress response (22). Multiple reports indicate higher activity of the brain noradrenergic system during stress, which has been implicated as a contributor to a decrease in NE neuronal stores (18, 19, 23). Correspondingly, others have reported an elevated level of extracellular NE (24). Stress induces activation of LC by CRF via projections from hypothalamus as well as amygdala (25, 26). Intracerebroventricular injections of CRF lead to elevated firing of the LC neurons and apparent release of NE in the terminal projection regions like amygdala, hippocampus and hypothalamus. Moreover, NE has been found to stimulate CRF containing cells in the hypothalamus (to stimulate the hypothalamic-pituitary-adrenocortical axis) as well as amygdala (21). This has been proposed to generate a positive feed-forward loop during stress, which if not regulated might give rise to various adverse symptoms such as depression, anxiety, panic reaction and hyperarousal (27). Moreover, there are reports of stress-induced increases in release of NE within LC (28, 29).

In LC, CRF and NE act predominantly via two G protein-coupled receptors (GPCR), the corticotrophin-releasing factor type 1 receptor (CRF_1-R) and the $alpha_{2A}$-adrenoceptor (α_{2a}-AR), respectively. Corticotrophin-releasing factor activates CRF_1-R in the LC to increase its firing (30). On the other hand, NE acts on axonal/dendritic α_{2A}-AR to inhibit firing of the LC neurons and acts on presynaptic α_{2a}-AR (on recurrent collaterals and terminals of afferent projections to LC) to modulate release of neurotransmitters (29). Both the agonist-occupied

CRF_1-R and the α_{2A}-AR are preferentially phosphorylated and desensitized by G protein-coupled receptor kinase 3 (GRK3). This leads to GRK3-mediated homologous desensitization of these receptors (31-35). In most brain regions GRK2 expression is higher than GRK3. However, LC is one of the few brain areas where GRK3 expression is higher than GRK2 (36). Evidence from our lab and others implicate a dominant role of GRK3 in the modification of signaling of the G protein-coupled receptors which are found in LC and through which neuromodulators NE, CRF and dynorphin exert their effects (31-33, 37-41). Clinical studies also have indicated that a single nucleotide polymorphism (SNP) in the promoter region of the GRK3 gene is observed in a subset of patients with bipolar disorder. The SNP is accompanied by low GRK3 levels in the lymphocytes derived from those patients. These patients also demonstrated an elevated susceptibility for stress-induced relapse of their bipolar disorder symptoms (42-45). There are other reports of lower levels of membrane GRK3 without any change in levels or mRNA of GRK2, in the cortex of patients diagnosed with bipolar disorder as compared to the controls (46). Moreover long-term treatment with therapeutically relevant doses of the mood-stabilizers lithium and carbamazepine resulted in increased membrane GRK3 but not GRK2 in the frontal cortex of rats (47). All these reports suggest a potential role of GRK3 in adaptation to stress by desensitization and termination of signaling of GPCRs like CRF_1-R and α_{2a}-AR, in the face of relentless stimulation by the

neurotransmitters during stress. Hence, it is possible that diminished levels of GRK3 in LC will cause dysregulation of GPCRs, leading to hyper-responsiveness of LC and to adverse behavioral effects.

The processes of adaptation of neurotransmitter/hormone signaling are part of a larger process that constitutes an individual's ability to succumb or adapt to stress. A critical shortcoming in our current knowledge relates to our understanding of the factors that distinguish those that are susceptible to the adverse psychological consequences of stress from those who are less susceptible, or stress resilient. Amongst the various animal models used to evaluate antidepressant drugs, the very commonly used Learned Helplessness paradigms offer the opportunity to identify, compare and contrast stress-susceptible and stress-resilient populations. In the classical Learned Helplessness paradigm, rodents are subjected to uncontrollable and inescapable stress in the form of electric shock, followed by testing their performance in an escape test in a situation involving an escapable milder shock. Rodents that give up trying to escape or showed increased latency time for escape as compared to the controls are considered Learned Helpless and the behavior is called learned helplessness (LH). Alternatively, the animals that escape similar to unstressed rats are termed Non-Helpless (NH). Thus, this model identifies stress-susceptible and stress-resilient populations and provides an opportunity to investigate the variations in the LC levels and functions of α_{2A}-AR, CRF_1- R and

GRK3 between the stress-susceptible and stress-resilient populations of rats. In addition, the consequences of exposure to single and repeated uncontrollable and inescapable stresses can be studied and compared.

It is not known if exposure to inescapable and uncontrolled stress other than the electric shock, such as forced swim stress, will induce LH behavior upon subsequent testing in an escape paradigm. The forced swim stress paradigm incorporates subjecting the animals to swimming in 30 cm deep water, thus providing an inescapable and uncontrolled stressful condition (48, 49). The forced swim paradigm is a commonly used method to assess antidepressant drug action. It has the advantage of allowing different behavioral readouts for assessing drugs acting via noradrenergic and serotonergic systems (50). However, forced swim is not typically used as a source of uncontrollable, inescapable stress after which animals are then subjected to other behavioral assessments like escape behavior.

STATEMENT OF PROBLEM

Adverse behavioral consequences of stress have been associated with hyper-responsiveness of the LC in response to stress (21, 27, 51-53). However, the exact reasons leading to this increased activity of LC during exposure to stress have not been elucidated. There is a stress-induced activation of α_{2A}-AR and CRF_1- R in the LC that increase and decrease LC neuronal firing, respectively. However, it is not known, on exposure to repeated stress, how the functions of the receptors change. The first goal of the present study was to validate the forced swim stress, a model of stress other than the electrical shock paradigm, as a more physiologically relevant model that can induce LH behavior A second goal of the present study was to investigate the changes in cellular mechanisms, involving α_{2A}-AR, CRF_1- R and GRK3, associated with changes in behavior of rats on exposure to single and repeated forced swim stress and to investigate the changes in function of the α_{2A}-AR in LC of rats exposed to repeated forced swim stress. Finally, the study evaluated the efficacy of desipramine in the prevention of both repeated forced swim stress- induced LH behavior and in the prevention of repeated stress-induced changes in α_{2A}-AR, CRF_1- R and GRK3 in LC.

The completion of the study serves the following purposes: 1) it validates a novel model other than electrical shock as a method to induce LH behavior; 2) it sheds light on the difference in neuronal levels of α_{2A}-AR, CRF_1- R and GRK3 in LC and their association with behavioral changes on exposure to single and repeated stress; 3) it demonstrates changes in functions of α_{2A}-AR during exposure to repeated stress; 4) it adds to our knowledge of the role of GRK3 in specific brain regions relevant to stress-induced behavioral changes.

2. LITERATURE REVIEW

2.1. Stress – the beneficial evil.

Stress can be defined as any possible challenging scenario or disturbances that disrupts the normal psychology and physiology (10). In general living creatures tend towards stability and try to maintain a homeostatic condition (54). Beneficial or good stress has short temporal limits, which can be taken control of and can be helpful for a living being's survival. The stress response is characterized by adequate turning on of the autonomic and hypothalamic-pituitary- adrenal axis and behavioral responses to stressful stimuli. This also may lead to the activation of the "flight-or-fight" response to an adverse or life-threatening situation. This response is mediated by the release of various molecules (neurotransmitters / hormones) which, acting via various receptors or ion-channels, produce a wide range of downstream effects. These engagements of the body to undertake steps to preserve stability in the face of adverse challenges are termed "allostasis". The sum total of these effects help the animal to "adapt" and react to the new situation in order to perform activities like, running away or fighting a predator, waking up from the bed in the morning or moving up stairs (55). However, if the response to a challenging condition is not adequate enough or if

the stress occurs repeatedly then it might lead to pernicious conditions contributing to elimination of the living creature or at least adverse health consequences (10). Thus when the responses to stress are chronic, the effects of the intermediary neurotransmitters and hormones linger over extended periods and, especially if there is not adequate regulation, lead to damage to the body and brain. This is termed “allostatic load or overload” (10). Stress has been associated with the prevalence of and the precipitation of many neurological and psychiatric disorders (56). It has been noted that there is an increase in levels of NE and its metabolites in cerebrospinal fluid of patients suffering from depression (57). This finding is supported by another finding where there was an increased level of tyrosine hydroxylase (TH) in the LC of depressed suicide patients (58). This point to the possibility that LC basal activity might be increased in depressed patients.

2.2. Stress response – the spatial and temporal dynamics.

Stress implicates modification of behavior and future reactions in response to a perceived or real adverse condition. This engages alteration of neuronal activities within different brain regions that are responsible for learning, cognition, mental state and autonomic consequences. Depending on the type of stress,

diverse mediators influence particular brain regions acting at characteristic temporal levels.

Occurrence of an adverse event is followed after a brief time lag by increases in the levels of monoamines like noradrenaline, dopamine and serotonin in particular regions of the brain. This happens due to secretion of these monoamines from nerves originating in the brain regions involved in the assessment of the stressful happenings or due to stimulation of the sympathetic nervous system. The degree of stimulation of these circuits involving the monoamines is influenced by a variety of determinants such as gender (59), time point in the circadian cycle (60) and the individual's power to direct the degree of stressor or repeatability of the stress (61). The literature reveals an increase in the levels of monoamines following exposure to stress in various brain regions including amygdala, prefrontal cortex, nucleus accumbens and LC. However, the significance of this phenomenon is dependent on the affinity and location of receptors for these monoamines in various brain regions. Therefore, every monoamine has a function to perform in the whole cascade of events, depending upon where and when they are released, the target receptors present in that location and the affinity of monoamines for the local receptors. Monoamine effects are prompt in response to stress exposure and in the range of minutes. Monoamines predominantly mediate their actions via GPCRs that rapidly stimulate the signaling cascades further down the line. These cascades modify

the activities of the nerves on which the receptors are located and these modifications further translate into behavioral consequences. It has been found that elevated release of NE in specific brain areas resulted in transfer of focused attention to non-specific scanning of the surroundings to respond to the adversity (62). Another monoamine, dopamine, on exposure to stress, is increasingly released in prefrontal cortex, and has been associated with improved judgment of risk (63). On the other hand serotonin, has been associated with attenuating the anxiety following stress-exposure (64). Hence, the cumulative effects of monoamines are to formulate responses that help the animal cope with the initial period of the adverse conditions.

Responses to stress also are mediated via various neuropeptides such as corticotrophin-releasing factor (CRF), vasopressin, orexin, ghrelin and dynorphin. There also are others, like, oxytocin and neuropeptide Y, which oppose the stress response pertaining to particular circumstances. Moreover, neuropeptides like galanin and substance P have been implicated in anxiety and dysphoria associated with stress (65).

CRF and vasopressin are two important neuropeptides that play vital roles in eliciting rapid stress responses pertaining to behavior and metabolism. Activation of the hypothalamic-pituitary-adrenal (HPA) axis plays a pivotal role in the neuroendocrine aspect of the stress response. This is initiated by synthesis of CRF and vasopressin in the paraventricular nucleus of hypothalamus and

secretion into the portal vessel system. CRF then stimulates production of pro-opiomelanocortin (POMC) in the anterior pituitary and the subsequent release of POMC products such as adrenocorticotropin hormone (ACTH), opioids and melanocortins (54). ACTH acts on the adrenal cortex resulting in release of cortisol (in human) and corticosterone (in human, rats and mice). Numerous inputs from the limbic region (stimulated during stress and affecting emotion) and the brain-stem region (which provides a pathway for the stimulus from viscera and senses) activate the paraventricular nucleus. Other related peptides include urocortin I and II whose actions are mediated via receptors for CRF, although the action is mediated in a slower mode than actions of CRF itself. CRF acts via corticotrophin-releasing factor 1 receptor (CRF_1-R) and corticotrophin-releasing factor 2 receptor (CRF_2-R) that are GPCRs. Within seconds of its secretion, CRF predominantly acting via CRF_1-R, exerts its effects on the target nerve cells, establishing it as one of the important mediators of the rapid stress response in the temporal dynamics (66), (67).

Corticosteroids form another important arm of the stress response and are transported to all body organs by the circulatory system. The corticosteroids synchronize activities of the central nervous system and the body, leading to coordinated responses to stressful stimuli, contributing to the establishment of a new equilibrium and facilitating subsequent recuperation. Corticosteroids mediate both the fast initiation phase and the slow termination phase of the stress

responses by acting via mineralocorticoid (MR) and glucocorticoid (GR) receptors. MRs are found abundantly in neurons of hippocampus, and lateral septum, and in limited density in amygdala, PVN, and LC whereas the location of GRs is ubiquitous including neurons and glia. The MR mediates the course of judgment and initiation of the stress response. GRs mediate the cessation of the primary stress response, the immune reactions, the assembly of substrates for energy and the process of recuperation, in addition to playing a role in learning and memory processes.

The coordinated action of the many and varied mediators in the stress response occurs through the activation of their receptors in various brain regions and in different temporal domains. Some of these important brain regions include hippocampus (CA1, CA3 and dentate gyrus), prefrontal cortex, amygdala (medial amygdala, basolateral amygdala and central amygdaloid nuclei), hypothalamus (paraventricular nuclei, PVN), dorsal raphe nuclei (DRN) and LC, all of which have specific roles to play in the neural circuits of the stress response. Hippocampus has been implicated as the site for control of the memory and learning process following a stressful incident (68, 69). Researchers also have implicated hippocampus as the region critical for stress-specific inhibition of the HPA axis by means of attenuating glucocorticoid release in rats as well as humans (23, 68, 70). Hippocampus also regulates autonomic tone (71). The medial prefrontal cortex also contributes importantly. The prelimbic prefrontal

cortex suppresses psychological stress-induced HPA axis activity and restricts the time period of glucocorticoid release (72-74) whereas the infralimbic prefrontal cortex triggers emotional stressor-induced autonomic and HPA axis responses (74-76). In addition, medial prefrontal cortex is associated with elimination of conditioned fear and the voluntary control of aversive emotion (77). The amygdala is associated with adding an “emotionally tag” to the memories of threatening stimuli in order to prioritize them to be stored in the hippocampus which is involved in the formation of the new memories (78).

2.3. LC and stress.

Dysregulation of the central noradrenergic system has been widely believed to be one of the prominent factors engaged in the development of the adverse consequences of stress that includes depression and other affective disorders (79). Previously, most studies focused on investigating noradrenergic function in areas that are supplied by noradrenergic neurons. However, it is logical to consider the role played by the origin of these noradrenergic supplies and recently many have focused in that direction. The locus coeruleus (LC) is a pontine nucleus comprising a compact assemblage of homogenous noradrenergic neurons, located adjacent to the fourth ventricle (80). LC forms the major source of noradrenergic input to diverse regions in brain and spinal cord

and is the exclusive NE supplier to cortex and hippocampus, the regions that are responsible for cognitive and behavioral responses to stress and formation of memory. In turn afferent innervations from a broad range of brain areas influence LC neuronal activity. Thus, one of the main roles of LC is the all-round preparation of the brain to responds to happenings in the outside and inside the body (81). One defining characteristic of LC neuronal firing is that it displays two different modes of electrical activity: tonic and phasic. Tonic firing is associated with arousal state and features a low frequency, continuous, and regular firing of neurons. In contrast, phasic firing is characterized by 2 – 3 short bursts followed by a quiescent period. Stimulation of LC results in desynchronization of the electroencephalographic pattern of electrical activity in the cortex and initiates theta oscillations in the hippocampus, both of which are considered signs of arousal (82). The low tonic mode of LC is associated with lethargy, detachment from the surroundings (decreased need for alertness), and depleted reaction to a sensory provocation compared to the moderate tonic mode to respond to stimuli (a particular level of tonic activity of LC neurons is needed). The phasic activity is coupled with a synchronized firing pattern of the LC neurons. This is due to electrotonic coupling of the dendritic regions of the neurons by means of gap junctions and in the pericoerulear area (83). By comparison the tonic pattern of activity of LC neurons is characterized by disruption in this electrotonic coupling (84). The degree of phasic mode discharge is influenced by the tonic mode. The

phasic mode is maximum when the tonic mode of discharge is at moderate levels. However the phasic mode is depleted when tonic discharge is significantly reduced (such as during sleep) or significantly increased above the moderate level (when there is aversive provocation) (84, 85). This can be envisioned as an inverted U-shaped relationship between phasic and tonic firing. The high tonic discharge pattern, when associated with a decreased phasic mode, signifies a transition from focused attention accompanying normal behavioral tasks to a general scanning of the environment. This indicates an adaptation towards preparing for or facing a stressful challenge and contributes to the transition from a reaction pertaining to a task to a reaction pertaining to a sensory provocation.

LC is regularly activated by different stressful stimuli. Evidence supporting this includes the stress-initiated elevation of NE turnover and metabolites in various brain areas for which the LC is the only source of NE (like cortex and hippocampus) (86-88). This was confirmed by investigations employing microdialysis which found elevated extracellular levels of NE in hippocampus on stress exposure (89). Moreover, studies demonstrate elevated mRNA and protein for TH or c-fos in LC during exposure to restraint stress, shock, hypotensive challenges, swimming, immune stress and psychosocial challenge (90-103). In turn LC is influenced by diverse afferents from various brain areas. A vital excitatory afferent projection to LC comprises the glutamatergic inputs from the paragigantocellularis nucleus (104). A dominant inhibitory afferent projection

to LC constitutes the GABAergic inputs from the prepositus hypoglossi and areas attached to dorsomedial regions of the medulla (105). Studies have pointed towards corticotrophin-releasing factor (CRF), acetylcholinergic and serotonergic afferent inputs to LC, in addition to innervations by neurons containing substance P and enkephalin (106-109). However, the most important input to LC that is related to stress is of neurons containing CRF. CRF inputs to LC originate from diverse brain areas like amygdala, Barrington's nucleus, and paraventricular nucleus of hypothalamus (110). A dense supply of CRF releasing nerve fibers have been identified both in the LC core (limited innervations) and periceorulear region (high innervations) (111). Studies have demonstrated both direct as well as indirect effects of CRF on LC (112). CRF nerve endings in LC are co-localized with glutamatergic and enkephalin-secreting ones (113, 114). This points toward the fact that all of the inputs, directly or indirectly, play roles to influence the LC-NE system. Stimulation of the LC by CRF is distinct from the stimulation produced by glutamate. The highest magnitude of stimulation of LC by CRF is lesser than the highest magnitude of stimulation of LC by glutamate. Also, the stimulation of LC by CRF is less gradual in onset and has a more extended period of action than the stimulation caused by glutamate (67). Studies indicate that CRF from afferent nerve terminals is secreted in the LC during stress (115). Evidence shows that this CRF acts directly on the LC neurons to stimulate it and thus CRF plays a neurotransmitter role in LC (30). This is different from the

neurohormonal role of CRF, where it is released from the paraventricular nucleus of hypothalamus and moves via the portal system to the median eminence to stimulate the stress-initiated release of adrenocorticotripin during stress (116).

Immunohistochemistry has indicated the presence of various opioid receptors in LC, namely, mu-opioid receptor (μ-OR) that are found post-synaptically and delta (δ) and kappa (κ) opioid receptors (OR) which are found on the terminals of nerves supplying CRF, dynorphin and glutamate to LC (22). The nuclei Paragigantocellularis and prepositus hypoglossi provide predominant innervations of opioids to LC (109, 117, 118). Enkephalin acting via μ-OR hyperpolarizes LC neurons via activation of inwardly rectifying potassium channels (GIRKs) (119). Opioids preferentially attenuate the tonic discharge of LC without influencing the phasic discharge (120). This would help to shift the firing from the high tonic mode to the phasic mode. It also has been found that morphine initiates synchrony in the oscillatory firing pattern of LC, and this is blocked by naloxone (121). This implies an interplay by CRF and opioids in that, while CRF promotes tonic discharge in order to increase arousal and scanning activities, opioids favor the phasic discharge to facilitate “focused attention” and to continuance of normal activities (67). Opioids also appear to play a role in returning the nature of neuronal discharge to pre-stress levels after termination of the stress (122). Antagonists for the κ-opioid receptor block immobile behavior in the forced swim test and attenuate escape deficits in learn-helplessness (123-

125). Evidence shows expression of κ-opioid receptors on the nerve endings of excitatory CRF or glutamatergic neurons, pointing towards the fact that κ-opioid receptors help in limiting different neuronal inputs to LC (22).

In addition to the reciprocal and varied interactions that have been reported between LC and other stress response-associated brain regions, there is another observation that supports the importance of LC in stress responses and stress-related disorders like depression. Many investigators have suggested LC is involved in modifying behavior, pharmacology, physiology and anatomy during the behaviors associated with the symptoms of depression as well as being involved in the actions of antidepressant drugs (79). Disruption of LC abolishes the action of desipramine on performance of rodents in the Porsolt forced swim test, an test used to screen anti-depressant drugs (126). The importance of the LC in the action of antidepressant drugs also is underlined by the fact that this region contains a substantial quantity of NE transporters through which tricyclic antidepressant drugs exert their actions (127).

2.4. CRF_1-R signaling in LC and stress

CRF elevates spontaneous firing of LC neurons when administered both *in vivo* and *in vitro* (30, 128). The action of CRF in LC is mediated by CRF1-R, which predominantly activate the guanosine triphosphate (GTP) binding protein

αGs, resulting in increased cyclic adenosine monophosphate (cAMP) production via adenylyl cyclase and also stimulation of protein kinase A (PKA) (129, 130). CRF receptor antagonists can block the increase in LC firing rates induced by intracerebroventricular (i.c.v) administration of CRF (53). Stress increases secretion of CRF in LC (67) and numerous studies have indicated changes in CRF receptor signaling in LC during stress. Factors that have been implicated in regulating the sensitivity of LC to stress include previous exposure to stress, prior opioid use and gender (67). CRF-evoked LC activation is enhanced following chronic exposure to cold stress (131). Additionally, studies report that exposure to a single swim stress selectively alters LC sensitivity to CRF (132). For example, a single swim stress-induced increased the coupling of CRF_1-R and αG_s, which indicates an increased sensitivity of CRF-mediated electrophysiological responses (133, 134). Furthermore, when CRF was administered i.c.v. in rats exposed to a single electric footshock stress, the dose-response curve for CRF, relating CRF dose to LC neuron firing, shifted to the right, indicating a decreased sensitivity to CRF (53). However, after exposure of rats to repeated daily footshock stress sessions for 5 days, the CRF dose-response-curve in LC is shifted to the left with a diminished maximal response. This leftward shift would mean that lower doses of CRF that would normally be ineffective, could now increase activation of the LC-NE system (53). It was speculated that this increase in sensitivity of LC might be associated with

symptoms like hyperarousal and sleep disorders which are some of the features of stress-induced psychiatric diseases like depression (53). Interestingly though, while various alterations in the sensitivity of LC to CRF were observed after various kinds of stress (footshock, immobilization and forced swim), prior stress exposure had no effect on the basal discharge of LC (21). The lack of effect on basal discharge would suggest that endogenous CRF does not tonically regulate basal discharge of the LC and this is supported by the observation that CRF receptor antagonist administration does not alter LC spontaneous firing in vivo (135). Gender also is a factor regulating LC responsiveness to CRF, with unstressed female rats showing higher sensitivity to CRF (136). The concentration response curves for CRF-induced LC neuronal discharge is shifted to the left in unstressed female rats compared to unstressed male rats. In addition, exposure to a single hypotensive stress caused a greater increase LC firing in female rats compared to male rats (136). Surprisingly however, prior exposure to a bout of shock or swim stress enhances LC responsiveness to CRF in male rats but not in female rats (136). There also have been reports of enhanced sensitivity of LC neurons to CRF in rats chronically administered morphine (137). This may be relevant to opioid abuse, a condition that is associated with stress (137).

Several of the above reports point to differential effects of exposure to a single vs. repeated bouts of stress on the CRF_1-R signaling in the LC neurons.

CRF biases the LC firing towards an elevated tonic mode and diminished phasic one. This has been purported to result in increased awakening, switching from a focused attentive behavior towards a general behavior that scans the surroundings (85, 138). This response can be considered as the development of an allostatic state mediating adaptation to the single or acute stress exposure. However, if there is not a proper termination of this response after cessation of the stress or if the stress exposure is repeated, then these changes may not be positive adaptive changes and may manifest in the pathological conditions like depression. Hyperarousal symptoms also can develop. Thus it becomes important to study the changes in signaling of CRF_1-R in LC neurons, after exposure to single stress as well as after exposure to repeated stress. The present study is an effort in that direction to contribute to understand the cellular events that are involved in the differential changes of CRF_1-R signaling that accompany the behavioral consequences of single and repeated stress.

2.5. α_{2A}-AR signaling in LC and stress

In LC α_{2A}-ARs are located both pre-synaptically and post-synaptically. Presynaptic α_{2A}-ARs are located on the terminals of the afferent noradrenergic nerves from distant sites as well as on the recurrent collaterals of LC neurons (29). Postsynaptically α_{2A}-ARs have been found to be located on somatodendritic

sites on LC neurons (29). Stimulation of postsynaptic α_{2A}-ARs in LC by agonists NE, epinephrine or clonidine cause neuronal membrane hyperpolarisation and suppression of neuronal firing (139). Classically, actions of α_{2A}-AR are mediated via $G\alpha_{i/o}$ (a guanine nucleotide binding protein) mediated inhibition of adenylyl cyclase with subsequent reduction of cAMP production (140). However in LC the inhibitory action on neuronal firing is predominantly mediated by direct G protein regulation of inward rectifying potassium channels (GIRKs). Activation of these GIRKs cause enhanced outward potassium conductance and subsequent hyperpolarization of the neurons (141). Activation of presynaptic α_{2A}-ARs inhibits NE outflow from noradrenaline-releasing nerve terminals as well as recurrent collaterals. The α_{2A}-AR agonist clonidine inhibits release of NE in LC by 50% (142). On the other hand, the α_{2A}-AR antagonist idazoxan elevates NE release (29). Reports indicate that after activation of α_{2A}-ARs by clonidine or NE, or after a burst of action potentials in LC neurons induced by intracellular pulses, there is an inhibition of LC neurons caused by membrane hyperpolarisation which can be blocked by the α_{2A}-AR antagonist piperoxane (143).

Very little information is available regarding responses of α_{2A}-ARs to agonist after exposure to stress. Response to cumulative doses of the α_{2A}-AR agonist, clonidine (administered intravenously) are augmented with enhanced inhibition of LC firing in rats exposed to seven daily 2 hr sessions of immobilization stress (52). This indicates an increased sensitivity of α_{2A}-AR in

LC after exposure to repeated immobilization stress. Contrastingly, after exposure to repeated 14 days (throughout the day on each of 14 days) for of cold stress the dose-response curve for clonidine-induced suppression of LC neuronal discharge shifted to the right (144). This indicates a decreased sensitivity of α_{2A}-AR after exposure to repeated cold stress. Thus there are differential effects on sensitivity of α_{2A}-AR, depending upon the type of repeated stressor to which the animal is exposed.

Studies also have reported changes in levels of α_{2A}-AR in LC after exposure to stress. Acute cold stress of 1 hr duration increased [^{3}H] Idazoxan binding in LC which indicates an increase in levels of α_{2A}-ARs (145). Also, extended exposure of 4 hr to mild (cold) stress resulted in decrease in levels of α_{2A}-ARs compared to a more intense stressor (52, 145, 146). These reports indicate alterations in LC α_{2A}-ARs levels that were dependent on the duration (time of each stressor exposure session) of stress, repeatability (single or repeated) of stress and method used to produce (intense or mild). It is therefore difficult to draw a conclusion due to the diverse models of stress used in the studies previously reported. An additional important consideration is that it is impossible to know if the changes in receptor levels seen in these studies reflected presynaptic or postsynaptic receptors, or both. An increase in levels and activity of postsynaptic α_{2A}-AR will tend to increase inhibition of LC neuronal activity resulting in inhibition of noradrenergic output to projection areas. This will

facilitate a counter-regulatory dampening effect in the face of increased LC discharge during exposure to stress. On the other hand, an increase in presynaptic α_{2A}-AR will result in blunted NE release in LC from the collateral projections on LC cell bodies and dendrites. This blunting of NE release during enhanced LC neuronal activity would be expected to enhance the response to excitatory neurotransmitters like CRF.

This study explores how the level and responsiveness of α_{2A}-AR in LC change in relation to changes in behavior on exposure to single and repeated forced swim stress. It also tries to elucidate the mechanism underlying those changes. One of the highlights of the study is to observe the effect of repeated swim stress on function of the α_{2A}-AR to cause membrane hyperpolarisation in LC neurons. Thus the study will add to the knowledge of how noradrenergic neurotransmission is regulated on exposure to forced swim stress and the role α_{2A}-AR play.

2.6. Regulation of levels of GRK3 and signaling of α_{2A}-AR and CRF_1-R in neurons of LC.

GRK3 preferentially regulates the signaling of α_{2a}-AR and CRF_1-R both of which are expressed on neurons of LC (31-33, 37). Thus there is a possibility that alteration in levels of GRK3 will alter the signaling of these receptors and

thus change the net activity of the neurons. (35). Previous studies from our lab have found that when GRK3 is increased 2 to 3 fold the epinephrine-induced downregulation of α_{2a}-AR was markedly more productive as compared to that when there was 2 to 3 fold increase in GRK2 (34). Also, the use of GRK3ct, which is the C-terminal part having the $G_{\beta\gamma}$ protein binding domain, blocked the down-regulation and desensitization of α_{2A}-ARs on exposure to epinephrine (34). GRK3 levels can be altered by diverse mechanisms at the levels of expression and stability of the protein. Previous works done in our lab using neuronal cell models have demonstrated that chronic CRF increases expression of GRK3 (147). Similarly, epinephrine treatment of neuronal cells elevated expression of GRK3 by simultaneously activating α_{2A}-ARs and beta$_2$-adrenoceptors (148). This was the result of an increase in transcription of GRK3 via stimulation of ERK 1/2 (147, 148). In addition, our lab has demonstrated the role of heat-shock protein 90 (HSP90) in GRK3 stability in the neuroblastoma BE(2)-C cells (35). Binding of HSP90 to GRK3 inhibits GRK3 degradation. Further it was demonstrated that calcium activated calpains increased GRK3 degradation and thus reduced GRK3 levels. Therefore, varieties of mechanisms regulate the normal levels of GRK3 and thereby might influence signaling of α_{2a}-AR and CRF_1-R.

2.7. GRK3, stress and mood disorders.

In brain, there are multiple G protein-coupled receptor kinases (GRK) expressed including GRK2, GRK3, GRK5 and GRK6. GRK2 and GRK3 represent a GRK subfamily of structurally similar GRKs that is widely expressed in brain. In most brain areas GRK2 is expressed at higher levels than GRK3. However, LC is one of the few brain regions where GRK3 expression is higher than GRK2 (36). Studies from our lab and many others indicate that GRK3 plays a dominant role in modification of signaling of a select group of GPCRs which are found in LC and through which various neuromodulators or neurotransmitters like NE, CRF and dynorphin exert their effects (31-33, 37-41). GRK3 contributes to the termination of receptor signaling by phosphorylating agonist-occupied GPCRs and initiating the process of desensitization which advances recruitment of arrestin. This process, in the face of relentless stimulation by neurotransmitters during stress, plays a role in curtailing GPCR signaling and thus in adaptation to stress (149). In addition to termination of certain signaling cascades, GRKs also are known to be involved in turning on of other signal cascades mediated via arrestin. Arrestins play a role in initiating signaling cascades like activation of mitogen-activated protein kinase (MAPK) by GPCRs.

There is ample evidence in literature to merit the investigation of role of the GRK3 and the GPCRs regulated by it in specific brain regions, in mediating

the behavioral consequences of exposure to stress. One of the receptors involved in stress responses and whose signaling is regulated by GRK3 is the kappa opioid receptor. Studies have demonstrated that there is attenuation in stress susceptibility when a kappa opioid receptor antagonist was administered in the nucleus accumbens. Stimulation of kappa opioid receptors by dynorphin in nucleus accumbens has been found to cause dysphoria and can contribute to aversive behavior. This effect is mediated by GRK3-induced phosphorylation of kappa opioid receptors and subsequent stimulation of p38 MAPK (41, 150-153). This may help to explain why GRK3 knockout mice exhibit decreased immobile behavior in modified forced swim stress, indicating a decreased behavioral despair and hence decrease susceptibility to stress. These observations have led to the suggestion that elimination of GRK3 protects against the adverse effects of stress (151, 152). Furthermore, it was found that when a dominant-negative GRK3 is over-expressed in nucleus accumbens, the dysphoric effects of a kappa opioid agonist were removed (151-153). However, this goes against the typical role of GRK3 to terminate signaling in face of receptor overstimulation during persistent stress. Contrastingly, there are reports demonstrating that in a model of spinal cord injury created by partial sciatic nerve ligation, kappa opioid receptor desensitization in spinal cord leads to neuropathic pain (154, 155). In GRK3-knockout mice, the GRK3-induced desensitization of kappa opioid receptors is absent and the neuropathic pain is eliminated. These studies point to

a fact that loss of GRK3 in different neuroanatomical regions will have different effects and it can be important to investigate region-specific effects of GRK3 loss as opposed to a total elimination of GRK3 in GRK3 knockout mice.

Recent studies from our lab have found that GRK3 levels in LC are reduced in some but not all rats after exposure to unpredictable and inescapable stress (156). These rats with reduced GRK3 levels also demonstrated escape deficits in behavior tests. In rats where there was no change in GRK3 levels in LC after stress the rats behaved like non-stressed rats (156). The decreased GRK3 levels also were accompanied by differences in regulation of α_{2A}- ARs and CRF_1-R in the LC compared to rats with no change in GRK3 or to the control rats (156). This implies that a deficiency of GRK3 in LC facilitates the adverse behavioral consequences of stress and that normal levels of GRK3 in LC might be playing a protective role during stress. Clinical reports have indicated the importance of GRK3 in psychiatric disorders. For example, a single nucleotide polymorphism (SNP) in the promoter region of the GRK3 gene has been observed in a subset of bipolar disorder patients and a low GRK3 level was found in the lymphocytes derived from those bipolar patients compared to the controls. The more reduced the GRK3 expression the more severe were the symptom in the patients. (42-44). Reports also indicate that the bipolar disorder patients with the SNP show increased susceptibility to relapses when exposed to stress (157). In another study of bipolar disorder patients, the frontal cortex

showed a significant reduction in GRK3 with no alteration expression of GRK2 (46). Long-term intake of the mood-stabilizing drug, lithium, has been reported to cause elevated GRK3 translocation to membrane and this was suggested to play a role in rectifying increased sensitivity of GPCRs during bipolar disorders (47).

All these observations taken together indicate that GRK3 may have an important role to play in modulating the response to stress. Thus in brain GRK3 can play a beneficial or adverse role during stress depending upon the specific brain region and the GPCR activated. The present study aims to investigate the role of GRK3 in the regulation of 2 important GPCRs, α_{2A}-ARs and CRF_1-R in LC after exposure to single and repeated swim stress. The association between this regulation and the behavioral changes caused by stress also will be studied. The α_{2A}-ARs and CRF_1-R are preferentially regulated by GRK3 and have inhibitory and excitatory influences on LC neuronal activity, respectively, which change during stress. There is a possibility that if the influence of GRK3 on CRF_1-R is more than α_{2A}- ARs then a decrease in GRK3 on exposure to forced swim stress will tilt the balance towards excitation. This will result in hyperactivation of LC neurons, a phenomenon that has been associated with various adverse consequences of stress.

2.8. Assessment of stress-susceptibility using the Learned Helplessness model and role of LC.

The LH paradigm is a model employed to predict antidepressant efficacy of a drug based on the behavior of rodents. In this model animals are first subjected to an inescapable, uncontrollable stress (generally electric shocks) in one or more sessions. Then the rodents are tested for performance in an escape test in response to milder footshock, where they have the opportunity to actively escape the footshock. Animals that give up trying to escape or show increased latency time for escape as compared to the unstressed rats, are described as exhibiting Learned Helpless (LH) behavior. Alternately, the animals that behave like the unstressed rats are termed non-helpless (NH). The increased escape latency can be attenuated or abolished by pretreatment with anti-depressant drugs such as tricyclic antidepressants or selective serotonin reuptake inhibitors. Studies have predominantly used two distinct variations of this model. In one variation, the same context is used for the initial stress and subsequent escape testing. Animals are subjected to unpredictable and inescapable footshock in the same shuttle-box where the escape testing is subsequent done. In the second variation, the animal is placed in a restrainer and the initial inescapable and unpredictable stress in the form of electric shocks is delivered through the tail. However, the subsequent escape testing is done in a shuttle-box (separate

context). Both variations of this model react to antidepressant treatments. However, there are points of divergence. One of the studies noted that in the first model, where there was a contextual connection between the stress paradigm and the escape testing atmosphere, the LH is accompanied by contextual learning and confounding fear (158). Additionally, exercise does not reverse LH in this model where the stress and escape testing are performed in the same context. However, they also reported that exercise training abolished the increase in the escape latency in the model where there is no relationship between the context in which the stressor is applied and the escape testing is done (158).

Development of LH behavior, which is a depression-like behavior, requires exposure to inescapable stress. Inescapable stress in the form of electric shock has been associated with an activating influence on LC neurons (159). Additionally, stress exposure and subsequent adverse consequences have been associated with an activation of a feed-forward circuitry (27). This feed-forward circuitry involves noradrenergic projections from LC releasing NE in amygdala and hypothalamus and reciprocal CRF projections from these regions to LC to release CRF (27). Development of LH behavior is blocked by administering CRF_1-R antagonists prior to exposure to inescapable shock (160). However, if the CRF_1-R antagonist was administered after inescapable shock and prior to testing for escape behavior, it did not block the development of LH behavior.

Also, the consequences of inescapable stress on behavior have been found to be reversed when the α_{2A}-AR agonist, clonidine was infused into LC (161). In contrast, piperoxane, an α_{2A}-AR antagonists, failed to block behavioral consequences of inescapable stress, when infused into the LC (161). Besides LC neurons, 5-hydroxytryptamine (5-HT) neurons of dorsal raphe nuclei also are activated on exposure to inescapable shock (159). However, the total percentage of LC neurons activated during exposure to inescapable shock was nearly 100%, a much higher than the percentage of activated neurons in dorsal raphe nuclei (159). Evidence also indicates higher excitability of LC neurons compared to dorsal raphe neurons on exposure to inescapable stress (electric shock) (159). Elevated activity of 5-HT dorsal raphe neurons have been suggested to be associated with behavioral deficits induced by inescapable stress (61, 162-164). Since LC projects to the dorsal raphe nuclei, elevated discharge of LC neurons might facilitate enhanced activity of dorsal raphe nuclei neurons. NE from LC neurons activates dorsal raphe nuclei via α_1-adrenoceptors. The behavioral consequences of inescapable tail shock were abolished by infusion of the α_1-adrenoceptor antagonist, benoxanthian, into the dorsal raphe nuclei prior to the inescapable shock (164-166). The importance of dorsal raphe nuclei in the escape deficits induced by inescapable shock also is supported by the observation that destruction of the dorsal raphe nucleus abolished the escape deficits (166). Although the importance of the part played by dorsal raphe nuclei

cannot be downplayed, collectively these observations suggest that LC activation contributes importantly to changes in dorsal raphe nucleus that are associated with the development of behavioral deficits on exposure to inescapable stress.

Congenital LH rats also can be used to elucidate the molecular mechanism involved in the behavioral impairment that can be modified by antidepressant drugs. These congenital LH rats are male Sprague-Dawley rats which demonstrated LH behavior and were preferentially inbred for multiple generations to obtain rats that demonstrated enhanced susceptibility to development of LH (167). These congenital LH rats demonstrated an elevated immobile behavior accompanied by an attenuated swimming and climbing behavior in the forced swim test, compared to the non-congenital LH rats (167). Thus they can be used as a tool to study stress-susceptibility in rats. To date only genetic screening studies have been performed in this model.

Comparatively few studies have been done to judge the global effects of uncontrollability and inescapability of stress, using stress paradigms other than electric shock, on behavioral and psychological impairments. All the aforesaid arguments highlight the importance of the study of role of LC in the development of behavioral deficits on exposure to inescapable stress.

2.9. Forced swim as a model of stress

Classically, the forced swim paradigm is the most widely used preclinical model to assess antidepressant drugs and is better known as forced swim test (FST) established by Porsolt and colleagues (168). The forced swim stress involves placing the rat or mouse in an inescapable cylinder of water deep enough for the animal not to be able to touch the bottom with paws or tail. Animals, when put into this condition, demonstrate initial active behavior trying to escape from the situation but after that they adopt an immobile posture and make just those necessary activities that keep their head above water. This immobile phase is considered as failure to maintain escape-oriented activities (behavior despair) or adopting a passive posture that detaches the animal from attempting to cope with aversive events (169). A modified forced swim test is now employed which permits differentiation of selective serotonin reuptake inhibitors (SSRI) and selective NE reuptake inhibitors (SNRI) (50) . This is done based on the fact that in addition to decreasing the immobility time, the SNRIs increase the climbing behavior while the SSRIs enhance the swimming behavior (50). There is a parallel duality that can be observed during modification of depressive behavior in humans. Agents affecting the noradrenergic system, like reboxetine, tend to enhance motivation, whereas, serotonergic drugs tend to be more effective at elevating mood.

Various modifications of forced swim have been used for purposes other than to screen the activity of antidepressant drugs. For example, forced swim has been used as an aversive stimulus by some researchers. Swim increases LC sensitivity to CRF without any alteration of LC spontaneous firing (132). In another study, three day repeated forced swim stress was used to assess the effect of repeated aversive stimuli on analgesia (49). This study demonstrated a progressive increase in immobility time from day 1 to day 2 to day 3 and this was accompanied by long-lasting hyperalgesia. Both the hyperalgesia and increase in immobility were attenuated by antidepressant pretreatment. Some authors have used forced swim to induce long-term depressive conditions in mice. Here they subjected the mice to forced swim in mild warm water in tubs (170). This was done for 15 min daily for 4 days and then one day every week. This caused an increasing attenuation of the distance that the mice would swim and was accompanied by an enhancement of the immobile phase. This condition continued unchanged over weeks (170). Importantly, there was no attempt in that study to determine if all mice were equally susceptible to the effects of swim stress. Researchers also have used repeated forced swim as an inescapable stressor and found that it enhanced rewarding actions and self-consumption of alcohol. This effect depended on Kappa opioid receptor signaling (171). In another study repeated forced swim stress (numerous trials and prolonged stress application from which animals cannot escape) was used to show its effect on

increased craving for cocaine, an effect that was mediated by dynorphin and kappa opioid receptor association (172). This indicates that repeated forced swim stress paradigms can cause aversive behavioral consequences of stress exposure. Thus, forced swim stress offers an inescapable stress paradigm that has been used in various modified forms to study effects of aversive stimuli in animals.

The literature also contains studies where the forced swim test paradigm was used to identify animals that show different depressive behavior. Thus Wistar-Kyoto (WKY) rats, which have been suggested to have an endogenous depressive character, were segregated into two groups based on the amount of immobility exhibited in the forced swim test; WKY most immobile (WMI) and WKY least immobile (WLI). The WMI and WLI animals are then selectively inbred to generate animals that show differential effects of antidepressants and thus can be studied to understand the molecular causes responsible for depressive behavior (173).

The above-described use of forced swim as a stress is different from the classical forced swim test, which has been used to screen antidepressant drugs since this involves only a single swim exposure. Only one previous study has used forced swim as a model of stress to examine the effects of the controllability of stress on behavior of rats in a subsequent shuttle-box escape task (174). They observed that the escape deficits produced in the rats after exposure to repeated

inescapable swim stress were of a much lower degree compared to that produced due to the inescapable electric shock. However in that study they used intermittent repeated swim stress by subjecting the rats to 100 intermittently spaced 5-second swims. The total duration of these swims was roughly 7 minutes and this was done in a single day. The authors hypothesized that an enhancement of the degree of escape deficits could be achieved by modifying the paradigm they have used by increasing the duration of repeated swims but there have been no subsequent reports testing this hypothesis. Using repeated forced swim stress, like restraint and tail shock, has the advantage that there is no contextual relationship between the initial stress and the escape task testing environments. Perhaps more importantly, the repeated forced swim stress offers a much more physiological stress than electric shock that may still enable the identification of stress-susceptible and stress-resistant populations within a cohort of rats.

3. METHODS

3.1. Animals

Male Sprague-Dawley rats, age 42 – 48 days, obtained from Harlan Industries (Indianapolis, IN, USA) were used. Free access to food and water were provided to the rats, which were housed in a humidity and temperature-controlled room (23°C) within the animal care facility on a 12hr light/dark cycle (lights 7 A.M to 7 P.M). The animals were acclimatized to the animal facilities first for 10 days and then additionally to the behavior room the last 5 days of this acclimatization before initiating the experiments. During the last 5 days the rats transported from their home cages in the animal care facility to the behavior core facility and were handled for 15 minutes daily. The University of Houston Institutional Animal Care and Use Committee approved the animal protocols followed in this study and all experiments were performed in accord with the National Academy of Sciences Guide for the Care and Use of Laboratory Animals, 8th edition (175).

3.2. Forced swim stress paradigm

For the forced swim stress (FS), a Plexiglas cylinder (21 cm diameter, 45 cm height, Lafayette Instrument Company, Lafayette, IN) filled with 30 cm water, ~25°C is used. The water level required the rats to swim or stay afloat without the tails touching the bottom of the cylinder. For the sham swim (SS) controls the level of water was 4 cm and the rats get wet but are able to keep all the four paws resting on the floor of the cylinder without struggling. After each swim session, rats were taken out, dried with a towel and placed in a cage with an overhead heating lamp until they were completely dry and then returned to their home cage. After each swim session the cylinder was drained of water, the cylinder cleaned and fresh water was added to the cylinder for the next rat. All forced swims were performed between 10 A.M to 2 P.M. The swim sessions were recorded using a digital camera mounted horizontally at the water surface level for scoring of the behaviors, namely swimming, climbing and immobility. Immobility was characterized as minimal or no movement of the paws and a floating position enough to keep afloat on the surface of the water. Swimming was characterized as horizontal movement across the surface of the water using both front and the hind limbs. Climbing was characterized as attempting to break the surface of the water using both forelimbs and hind limbs and against the walls of the cylinder. A blinded observer scored the video of the first 10 min of

swim stress each day, noting the predominant behavior (swimming, climbing or immobility) during each 5-second bin during the session. The total number of scoring counts for each behavior were determined and averaged for the non-swim control, sham swim control and forced swim stress groups.

3.2.1. Single forced swim stress

In this stress paradigm, rats were exposed to a single forced swim stress or sham swim in the cylinder for 10 min. Twenty-four hours after the last swim, rats were tested for escape behavior in the shuttle-box.

3.2.2. Three days forced swim stress

In this stress paradigm the rats were subjected to forced swim in the cylinder for 10 min on day 1 and 20 min on each of day 2 and day 3. The sham swim controls were put in 4 cm of water for an equal amount of time on each of the three days. Twenty-four hours after the last swim, rats were tested for escape behavior in the shuttle-box or prepared for electrophysiology.

3.2.3. Seven days forced swim stress

In this stress paradigm the rats were subjected to forced swim in the cylinder for 10 min on day 1 and 20 min on each of day 2, 3, 4, 5, 6 and 7. The sham swim controls were placed in the cylinder daily following the same protocol but were put in 4 cm of water. Twenty-four hours after the last swim, rats were prepared for analysis of proteins in brain.

3.3. Escape behavior testing

Tests for escape behaviors of the rats were performed in a shuttle-box that consisted of two chambers with a plastic wall between them. The plastic divider had a door so that the rats could escape from one chamber to another in order to terminate the shocks. To start, the rats were placed in one chamber that was consistent throughout the study. Rats were acclimatized for 2 minutes. The rats then were subjected to 5 fixed ratio-1 (FR1) trials in which the rats needed to cross over just once from one chamber to the other one in order to terminate the shock. The FR1 trials assess normal motor performance on application of foot shock. The FR1 trials were followed by a no-shock period of 5 min. Then the rats were subjected to 25 fixed ratio-2 (FR2) trials. In these trials the rats needed to cross from the first chamber to the second, and then return to the original

chamber in order to terminate the shock. The intensity of foot shock used was of 0.6 mA and shocks were delivered randomly at an interval of 30 – 90s between the trials with an average inter-trial interval of 60 s, using software from Med Associates (St. Albans, Vermont). Escape latency was defined as the time it took the animal to perform the task required to terminate the shock during each FR1 or FR2 trial. The maximum time allowed was 30 s. If the rat was unable to terminate the shock within 30 s then the shock was automatically stopped and escape latency was set at 30 s for that trial. Failure to complete the escape task within 30 s was considered an escape failure. The average escape latency and the percent (%) escape failures for the FR2 trials for each rat were used to quantify escape behavior.

The escape behavior assessment (tests done in shuttle-box) was performed 24 hrs after the last swim for all three groups, non-swim control, sham swim control and forced swim stress. The rats that were exposed to swim stress and showed impaired escape behavior compared to the controls in the escape testing were termed Learned Helpless (LH). They exhibit adverse consequences of exposure to stress and thus are considered stress-susceptible. The rats that after exposure to swim-stress performed similar to the controls in the escape task are termed NH and are considered stress-resilient.

3.3.1. Data analysis for escape behavior

Mean escape latency and mean percent escape failure for the fixed ratio-2 trials was calculated for each rat. K-means cluster analysis was performed on the data from the forced swim stress group and the resultant clusters were compared using t-tests. If the clusters were found to be significantly different from each other, comparisons of clusters to the control groups also were performed using One-way ANOVA, followed by Tukey's test. If one of the swim stress clusters exhibited a larger escape latency and larger % escape failures compared to the non-swim control, that cluster was categorized as LH. A cluster that showed escape behavior similar to the non-swim control was categorized as NH.

3.4 Behavioral tests for cross validation of single and repeated stress studies.

3.4.1 Open Field Test

The open field tests were conducted in a Plexiglas chamber (60 x 40 x 50 cm). The tests were performed in the behavior core room using 150 lux ambient light intensity. The rats were first acclimatized to the behavioral room for 1 hr before the commencement of the test. To start the test, the rat was put in the

middle of the chamber and allowed to move around in the chamber. To analyze the data, a central zone was designated by a 25 cm x 25 cm square area in the middle of the open field chamber. The time allowed for the test was 30 min. An Opto-Varimex Micro Activity Meter v2.00 system with integrated software (Optomax, Columbus Instruments; OH) recorded and quantified the movement and activities of the rat in the chamber. The chamber had eight infrared light emitting diodes and eight photo transistors, and when the rats undertook travelling or rearing activities the light beams were broken and the movement was detected and recorded simultaneously. The data accumulated in 3 min bins over the total test duration of 30 min. Locomotor activity was defined using parameters of vertical stereo and ambulant behavior. Exploration was identified by rotation, rearing and stereotypic activity. For analysis of anxiety behavior, several parameters were taken into account including the percent total time the rat occupied the central zone, the percent total number of entries and the percent total distance the rat moved in the central zone. More anxious behavior is indicated by reduced percent values of the aforesaid parameters. At the end of the 30min test, the rats were removed from the chamber and transferred to their respective home cage. The chamber was then sanitized using 70% alcohol to rid it of any animal scent from the last usage. Ten minutes intervals were given before placing the next animal in the chamber.

3.4.2. Elevated Plus Maze Test

Anxiety-related behavior was assessed using the Elevated Plus Maze (EPM) (Lochchi et. al., 2008; Consoli et. al., 2007). The maze is made of Plexiglass and has two open arms (50 x 10 cm) and two closed arms of identical dimension with walls 30 cm in height. The maze is place at a height of 50 cm from the ground. The arms are attached to a central platform (10 x 10 cm) and the total maze is uniformly illuminated at 30 lux. An overhead camera was used to record the movement of the rats. The animals were habituated to the test room for an hour before the test. The total time allotted for each trial was 5 min. At the start, the rat was positioned in the central part of the maze, facing one of the open arms. The number of entries made and the time spent in the closed and open arms and in the central area was manually noted from the videos. When all four paws enter into an arm, it is considered as an arm entry. At the end of each test the rat was transferred to their respective home cage and the maze was cleaned thoroughly using 70% alcohol and paper towels. A ten minute interval was given before testing the next rat. A decrease in open arm activity (duration and/or entries) is considered an indication of increased anxiety-like behavior.

3.5. Electrophysiology

3.5.1. Brain Stem Slice Preparation and Incubation

Sixteen to twenty-four hours after the last swim, rats were anaesthetized with isoflurane, the brains removed and placed in ice-cold dissection buffer (in mM: 229 Sucrose; 11 D-glucose; 1.25 $NaH_2PO_4.H_2O$; 25 $NaHCO_3$; 2.4 $CaCL_2.2H_2O$; 1.2 $MgSO_4.7H_2O$; 2.5 KCl; pH 7.4; osmolality 230 mOsml) for 1 min. A crude block of tissue of 5 – 8 mm length and containing major portions of the pons and cerebellum was dissected out. The rostral side of this block was glued to the floor of the vibratome slicing chamber and submerged in dissection buffer at 4ºC, equilibrated continuously with 95%O_2 and 5% CO_2. Then, 300µm coronal sections were made with the vibratome (Vibratome Sectioning System 3000, Warner Instruments, CT). Slices containing LC were identified using the 7^{th} cranial nerve and 4^{th} ventricle as reference points. Typically, three slices containing LC were obtained from each rat. These slices were immediately transferred to an incubation chamber containing artificial cerebrospinal fluid (ACSF) (in mM: 126 NaCl; 11 D-glucose; 1.25 $NaH_2PO_4.H_2O$; 25 $NaHCO_3$; 2.4 $CaCL_2.2H_2O$; 1.2 $MgSO_4.7H_2O$; 2.5 KCl; pH 7.35 – 7.37; osmolality 203 mOsml) and gassed with 95%O_2 and 5% CO_2 at room temperature (~25°C). The process of brain removal, slicing and transfer to ACSF was completed within 6 min. The

temperature of the incubation chamber was slowly raised from room temperature to 37ºC and the cells were allowed to recover for a period of 45 min at 37ºC. This process provided the healthiest slices that remained viable for a period of 7 hr. After the recovery period, a slice was transferred to the recording chamber which was perfused with ACSF saturated with 95%O_2 and 5% CO_2 and containing 1µM cocaine hydrochloride (Sigma-Aldrich, St. Louis, MO) and 100 nM prazosin hydrochloride (Sigma-Aldrich, St. Louis, MO). The superfusion ACSF was maintained at 35ºC throughout the experiment using an automatic temperature controller (TC-324B, Warner Instrument LLC, Hamden, CT). The circulating ACSF was not recycled. The slice was acclimatized to the recording chamber for 20 min before starting the experiment.

3.5.2. Recording

Whole-cell recordings were obtained from neurons of LC using a Multiclamp 700B amplifier (Molecular Devices, Inc., Sunnyvale, CA) and Digidata 1440A data acquisition system (Molecular Devices, Inc., Sunnyvale, CA). Patch-pipettes were fabricated from borosilicate glass (1.5 mm internal diameter; World Precision Instruments, Inc., Florida.) and pulled using a P-97 Flaming/Brown type micropipette puller (Sutter Instrument Company, Novato, CA). The electrodes were filled with an internal solution (in mM: 115 potassium gluconate; 20 KCl; 10

HEPES; 0.5 EGTA; 14 sodium creatine; 4.5 Mg-ATP; Na-GTP; 2mg/ml neurobiotin; pH 7.2 – 7.3; osmolality 280 – 290 mOsml). Electrodes having a resistance of 4 – 5 MΩ were used. After obtaining the patch, spontaneous firing rate was determined and only those cells that had a spontaneous firing rate of 0.5 to 3 Hz were selected for study. Then, membrane input resistance was determined. Input resistance also was determined at the end of the experiment. Data were used for the analysis only if there was not more than 15% change in input resistance from start to end of the experiment. The recordings for the experiments all were obtained in voltage-clamp mode (cells held at -60mV). For each drug concentration the increase in current was noted. The data was first processed and the currents were measured using software Clampfit 10.2 (Molecular Devices, Inc., Sunnyvale, CA).

Concentration-response curves to NE were determined and NE was applied into the superfusion ACSF using a syringe pump (Model 100 series; KD Scientific, Inc., Holliston, MA). By adjusting the concencentration of drug in the syringe and rate of infusion into the recording chamber the desired bath concentrations (0.3, 1, 3, 10, 30 and 100 μM) of NE were obtained. Norepinephrine (L-(-)-norepinephrine bitartarate salt monohydrate; Sigma-Aldrich, St. Louis, MO) was dissolved in ACSF as a stock concentration and applied through the syringe. The “dead-time” between switching on the syringe-pump and entry of drug into the chamber was approximately 40 sec. The effect

of NE was generally detected within 20 sec of entering the chamber and equilibrium was obtained within 2 – 3 min. The effect of each NE concentration was observed for total of 6 min before infusion was stopped. A 10 min washing was given before start of the next concentration. After recording of response to the highest NE concentration, 100μM, the α_{2A}-AR antagonist idazoxan (1 μM) (Idazoxan hydrochloride; Sigma-Aldrich, St. Louis, MO) was administered simultaneous with NE to exhibit that the NE responses were due to activation of α_{2A}-AR. In LC neurons, activation of α_{2a}-AR by NE causes hyperpolarization of the neuronal membrane via activation of outward potassium currents (139). Therefore, the effect of NE on membrane outward current was determined.

After each recording was completed, the recording patch-pipette was used to inject neurobiotin into the cell from which recording was done. The slices were submerged in 4% paraformaldehyde solution in PBS and fixed for 48 hrs. Neurobiotin staining was later developed using a Vectastain ABC reagent kit (Vector Laboratories, Inc. Burlingame, CA) and diaminobenzidine (DAB) (Vector Laboratories, Inc. Burlingame, CA) as per the manufacturer's protocol.

3.6. Desipramine Treatment.

First, the rats were acclimatized for 10 days as described above. Then desipramine (5mg/kg, bid) was administered for 11 days (Fig 1). Treatment was

accomplished using a physiological solution of desipramine hydrochloride (Sigma Aldrich, St. Louis, MI, USA), prepared by dissolving 5.7 mg/ml of desipramine hydrochloride in 0.9% saline with warming due to low solubility of the drug and followed by filtration sterilization. The solution was administered intraperitonealy (0.1ml/100g of body weight of rat) twice daily at approximately 7.30 am and 6.30 pm. The rats were subjected to forced swim on the 8th, 9th and 10th days of the drug treatment period. On the 11th day the rats were subjected to escape testing in the shuttle-box and on 12th day the brains were removed under anesthesia.

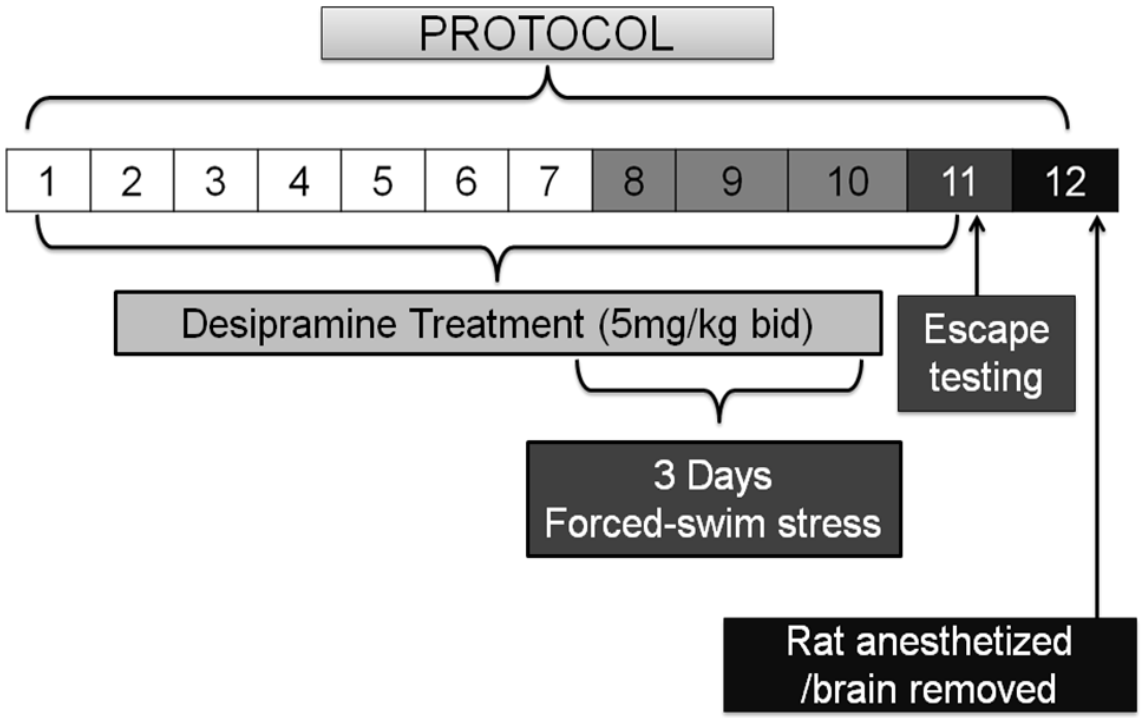

Figure 1: Time line for drug treatment, forced swim stress, escape behavior testing and removal of brain.

3.7. LC isolation and preparation of homogenates

The rats were anaesthetized with isoflurane (Nicholas Piramal India Ltd) and the brains isolated and stored at -80ºC until sectioning. At the time of sectioning the brains were allowed to stand at -20° C in a cryostat (Leica CM 1850, Leica Microsystems Inc., Bannockburn, IL) for 30 min. Brain stem was isolated by gross dissection using a coronal cut at 1.0 mm rostral to the cerebellum. The brainstem was then embedded into optimal cutting temperature (OCT) compound (Tissue Tek, Sakura Finetek USA, Inc. Torrance, CA) creating an OCT/tissue block at -20ºC. This block is then placed on the mounting disc of the cryostat and 300µm sections were cut. The LC was punched from these sections using 1 mm^2 tissue punches. The fourth ventricle and 7^{th} cranial nerve were used as reference points to locate the LC according to Paxinos and Watson (176). The collected disc-shaped punches were placed into pre-chilled Eppendroff® tubes. The tissues were suspended in 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). The tissue was homogenized by hand using a plastic dounce homogenization pestle. This homogenate was then boiled at 100ºC for 10 min, allowed to cool for another 10 min and then centrifuged at 10,000

rotations per min (rpm) for 10 min at 4^{o}C. An aliquot of the resultant supernatant was used for analyzing the protein content and the remaining supernatant was diluted using 4X sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 0.1 mg/ml bromophenol blue). This sample was then stored at -80^{o}C until western blot analysis.

3.8 Protein estimation

Protein concentrations in the brain homogenates were determined using a protein detection kit from Pierce (BCA, Pierce, Rockford, IL) consisting of protein assay reagent A and reagent B (177). Bovine serum albumin was used as a standard.

3.9. Western Blot Analysis

Proteins in the brain tissue homogenate samples (30 μg) prepared above were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins then were electrophoretically transferred (90V, 2.5 h,4°C) to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare Biosciences, Piscataway, NJ) . The membranes were blocked by placing them in 5% nonfat dry milk prepared in TBS-T (20 mM Tris-HCl (pH 7.6), 137 mM NaCl

and 0.1% Tween 20) for 1 hr. One set of membranes was probed with primary and secondary antibodies 3 times. Each probing consisted of incubation with primary antibody for 1 hr at room temperature, washing and reprobing of the membrane with species-matched secondary antibody conjugated with horseradish peroxidase (HRP) at room temperature for 1 hr and membrane imaging. Between each probing the membranes were stripped (stripping solution comprising of 62.5 mM Tris-HCl, pH 6.8, 1% SDS, and 100 mM 2-mercaptoethanol). After stripping the membranes were blocked and reprobed for the next protein. Sequentially, the 3 probings performed were for; 1) GRK3, 2) GRK2 and 3) simultaneous probing for TH as well as for Glyceraldehye-3-phosphate dehydrogenase (GAPDH), the later being used as loading control. Simultaneous antibody incubation was done for TH and GAPDH because antibodies against both proteins are mouse monoclonal antibodies. Thus, each membrane was stripped only twice. Membrane images of the immunoblots were obtained using a Fluorchem imaging system and Alpha Ease FC 4.0 software (both from Alpha Innotech Corp., San Leandro, CA) was used to determine intensities of the immunoreactivity band. Second set of gels/membranes were generated and probed 3 times for; 1) α_{2A} –AR, 2) CRF_1-R and 3) GAPDH. For quantification and comparison amongst samples band intensities for each protein were normalized to GAPDH. The concentrations, dilutions and source of various antibodies used in the experiments are shown in table1.

Table1: List of primary and secondary antibodies, dilutions and the sources used for western blotting.

Proteins	Primary Antibody	Secondary Antibody
GRK3	Polyclonal rabbit anti-GRK3 1:500 Santa Cruz Biotechnology Inc., CA	Goat anti-rabbit HRP 1:500 Santa Cruz Biotechnology Inc., CA
GRK2	Polyclonal rabbit anti-GRK2 1:500 Santa Cruz Biotechnology Inc., CA	Goat anti-rabbit HRP 1:500 Santa Cruz Biotechnology Inc., CA
TH	Monoclonal mouse anti-TH 1:1000 Millipore, Billerica, MA	Goat anti-mouse HRP 1:1000 Santa Cruz Biotechnology Inc., CA
α_{2A} – AR	Polyclonal rabbit anti-GRK3 1:300 Santa Cruz Biotechnology Inc., CA	Goat anti-rabbit HRP 1:500 Santa Cruz Biotechnology Inc., CA
CRF_1-R	Polyclonal goat anti- CRF_1-R 1:500 Santa Cruz Biotechnology Inc., CA	Donkey anti-goat HRP 1:500 Santa Cruz Biotechnology Inc., CA
GAPDH	Monoclonal mouse anti-GAPDH 1:1000 Millipore, Billerica, MA	Goat anti-mouse HRP 1:1000 Santa Cruz Biotechnology Inc., CA

3.10. Data analysis

3.10.1. Western Blots

The average of the ratios of the immunoreactive band intensities of the proteins of interest to GAPDH were analyzed between the groups using one-way ANOVA followed by Tukey's post hoc test or Student's t-test (Prism, Graph Pad Software, San Diego, CA). When $p \leq 0.05$, the differences between the groups were considered significant. Data is represented here as % change in Means ± S.E.M compared to non-swim control

3.10.2. Behavioral analysis

The data is represented as Means ± S.E.M. The means between the groups were analyzed using one-way ANOVA followed by Tukey's post hoc test or Student's t-test (Prism, Graph Pad Software, San Diego, CA). When $p \leq 0.05$, the differences between the groups were considered significant.

3.10.3. Electrophysiology

The increase in membrane current in response to different concentrations of NE, mentioned above, were measured using software Clampfit 10.2 (Molecular Devices, Inc., Sunnyvale, CA). The response to 100μM NE was considered the maximal response and set at 100%. Concentration-response curves for each cell were then constructed by plotting each increase in current as a percent of maximal response in the cell versus the log molar concentration of NE. Only data from cells where the responses to all NE concentrations could be obtained were included for analysis. The EC_{50} between the groups were compared using one-way ANOVA followed by Tukey's post hoc test or Student's t-test (Prism, Graph Pad Software, San Diego, CA). When $p \leq 0.05$, the differences between the groups were considered significant.

3.10.4. Cluster Analysis of behavior and biochemical parameters in single and repeated stress studies

Statistical software SAS 9.2 (SAS Institute Inc., Cary, NC) was used to perform independent k-means cluster analysis for biochemistry in LC (protein levels for GRK3, α_{2A}-AR, CRF_1) to identify two clusters in the data. Similarly k-means cluster analysis was used to find two clusters in the data from escape

behavior (two clusters have two different means). The cluster analysis here bifurcated the data into two subsets in such a manner that the all the data in a particular cluster is similar in certain manner, for example they are closer to one of the two means. To determine if the clusters determined separately on the basis of behavior alone (escape latency/escape failure) were different from each other and from the controls (non-swim control and sham swim control), ANOVA was used. When $p \leq 0.05$, the differences between the groups were considered significant. Similarly, clusters from biochemical analysis were compared with the controls using ANOVA. Then, it was analyzed how accurately the clusters based on biochemistry could predict the behavior of the animals in the escape task. For this the overlap between the clusters identified independently on basis of behavior and biochemical analysis was compared.

4. RESULTS

4.1. Single forced swim stress-induced changes in behavior and proteins

4.1.1. Single forced swim stress induced changes in escape behavior in shuttle-box.

The rats were subjected to a single forced swim stress and then, after 24 hr, tested for escape behavior in the shuttle-box. In the 5 fixed ratio 1 (FR-1) trials, which were used to test for any motor deficit in the rats, there were no differences between any of the groups; non-swim control (NC), single sham swim control (SSS) and single forced swim stress (SFS) (Fig 2 A, B). This ruled out any differences in locomotor function between the groups. When the rats were subjected to the fixed ratio 2 (FR2) trials, the single forced swim stressed rats did not demonstrate any significant differences in the escape behavior (mean escape latency or percentage escape failure), compared to either control group, the non-swim control or the sham swim control rats (Fig 3 A, B). However, when K-means cluster analysis was applied to the behavior data (mean escape latency and percentage escape failure from FR2 trials) from the SFS group, the analysis indicated a bifurcation of the data into two clusters (Fig 3 C, D). These clusters

were significantly different from each other. One of these clusters showed significantly impaired escape behavior (significantly increased behavior mean escape latency and significantly increased percentage escape failure) compared to either control group, the NC or SSS, and was termed Learned Helpless (LH). The escape behavior of the other cluster was not significantly different from the controls and was termed Non Helpless (NH).

The FR1 data of the LH and NH rats also were compared with the controls (Fig 2 C, D), but were not found to differ significantly. This indicated that in the FR2 trials, any differences between the groups were not due to locomotor deficits within the clusters of stressed rats. This rules out motor deficits as a potential cause for differences that might be observed between the groups in the FR2 trials.

FR1

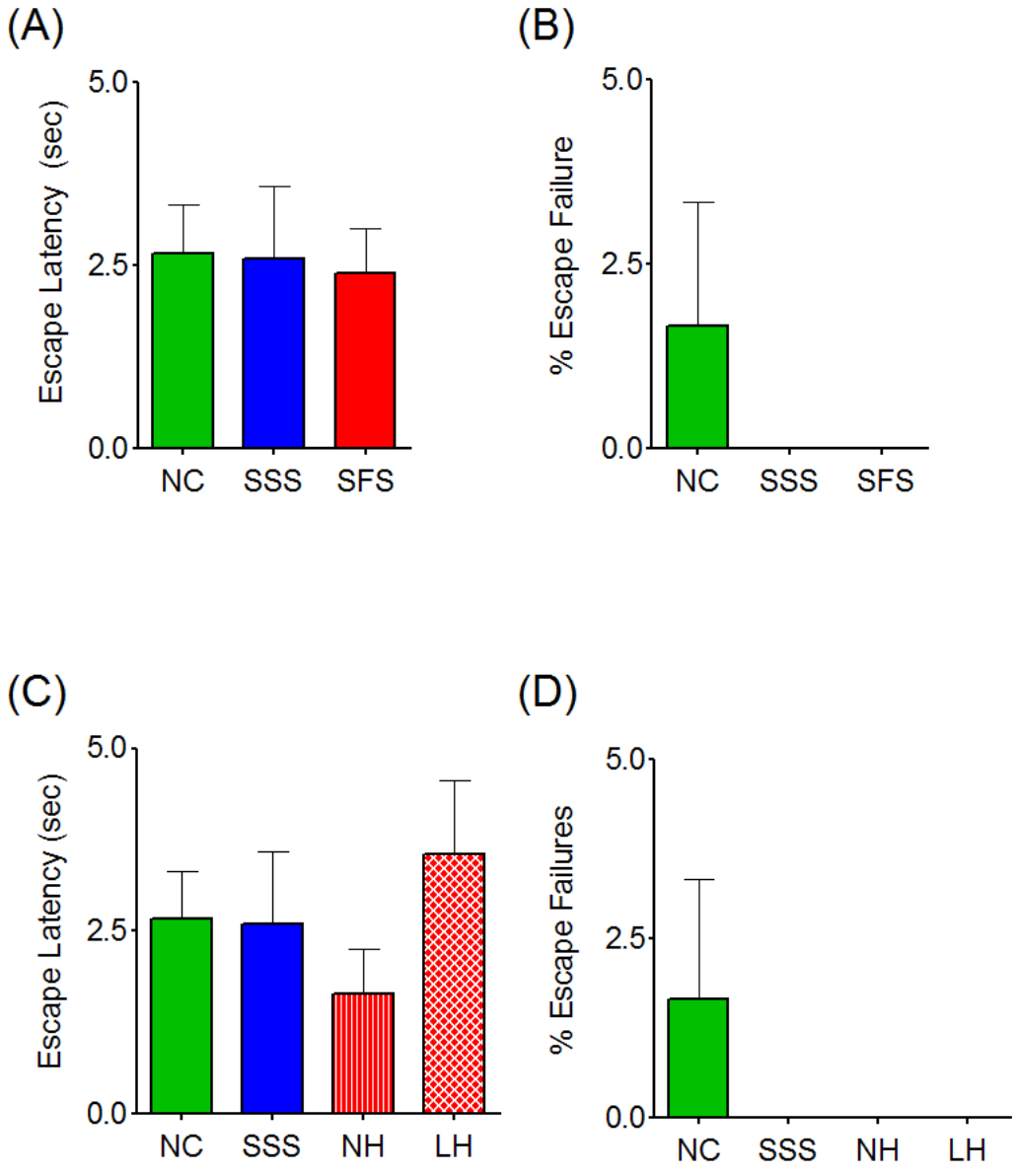
(A)
Escape Latency (sec)
5.0
2.5
0.0
NC
SSS
SFS
(B)
% Escape Failure
5.0
2.5
0.0
NC
SSS
SFS
(C)
Escape Latency (sec)
5.0
2.5
0.0
NC
SSS
NH
LH
(D)
% Escape Failures
5.0
2.5
0.0
NC
SSS
NH
LH

Figure 2: Exposure of the rats to a single forced swim stress did not demonstrate any motor deficits in the fixed ratio 1 (FR1) trials.
Rats were randomly assigned to three groups, named non-swim control (NC, n=10), single sham swim control (SSS, n=10) and forced swim stress (SFS, n=10) as described above. Twenty-four hours after the last swim all the rats were tested for escape behavior in the shuttle-box. First the rats were observed for any motor deficits using 5 fixed ratio 1 (FR1) trials. The FR1 data of the Learned Helpless (LH) and the Non Helpless (NH) rats as mentioned above were also compared with the controls (NC and SSS). No escape failures were observed in the groups where there are no bars.
Panel A – Mean escape latencies for NC, SSS and SFS rats for the FR1 trials. **Panel B –** Percentage escape failures for NC, SSS and SFS for the FR1 trials. **Panel C and D –** Mean escape latencies and percentage escape failures for LH (n=4) and NH (n=6) rats for the FR1 trials. Data are presented as mean±S.E.M.

FR2

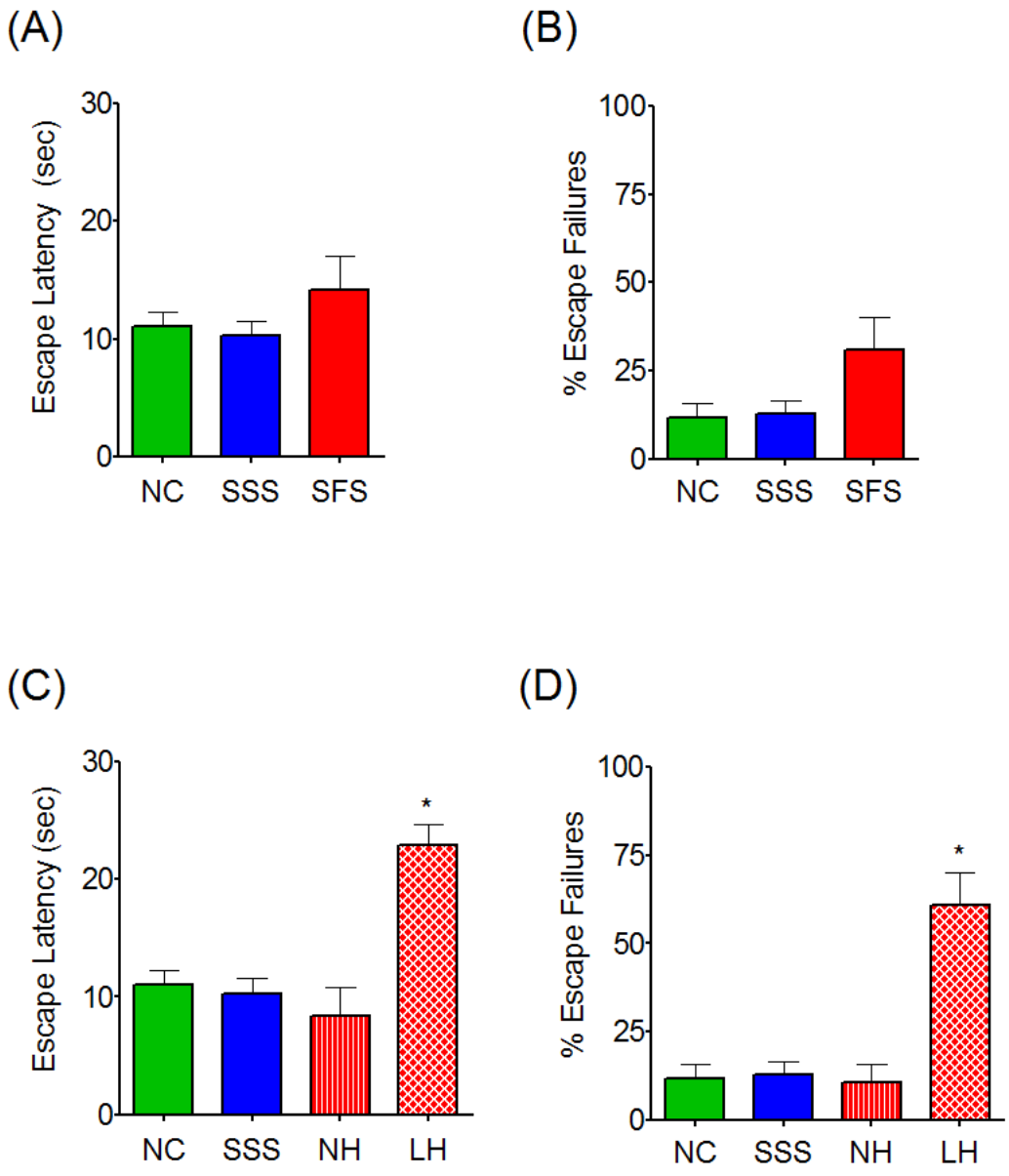
(A)
Escape Latency (sec)
30
20
10
0
NC
SSS
SFS
(B)
% Escape Failures
100
75
50
25
0
NC
SSS
SFS
(C)
Escape Latency (sec)
30
20
10
0
*
NC
SSS
NH
LH
(D)
% Escape Failures
100
75
50
25
0
*
NC
SSS
NH
LH

Figure 3: Exposure of rats to a single forced swim stress in rats resulted in two behaviorally different groups in the fixed ratio 2 (FR2) trials.
Rats were randomly assigned to three groups, named non-swim control (NC, n=12), sham swim control (SSS, n=10) and forced swim stress (SFS, n=10) as described above. Twenty-four hours after the last swim all the rats were tested for escape behavior in the shuttle-box subjecting each rat to 25 fixed ratio 2 (FR2) trials to observe the escape behavior as described above.
Panel (A) – Mean escape latencies for NC, SSS and SFS rats for the FR2 trials.
Panel (B) – Percentage escape failures for NC, SSS and SFS for the FR2 trials.
Panel (C and D) – Mean escape latencies and percentage escape failures for Learned Helpless (LH, n=4) and Non Helpless (NH, n=6) rats for the FR2 trials, as identified from k-means cluster analysis. Data are presented as mean±S.E.M. and * indicates significantly different from NC, SSS, and NH groups, $p<0.05$.

4.1.2. Open field test

The open field test allowed investigation of the locomotor and exploratory behavior of the NC, SSS control and NH and LH clusters within the SFS rats. There were no significant differences in the locomotor activity, assessed by total activity, between any of the groups (Fig 4A). Also, exploratory behavior of the rats, assessed by their rearing counts (Fig 4B), did not demonstrate any significant differences between NC, SSS, NH and LH rats. Anxiety behavior also was evaluated from the open field test by calculating the percentage of total time spent by rats in the central compartment. There were no significant differences amongst NC, SSS, NH and LH rats for this parameter. (Fig 4C).

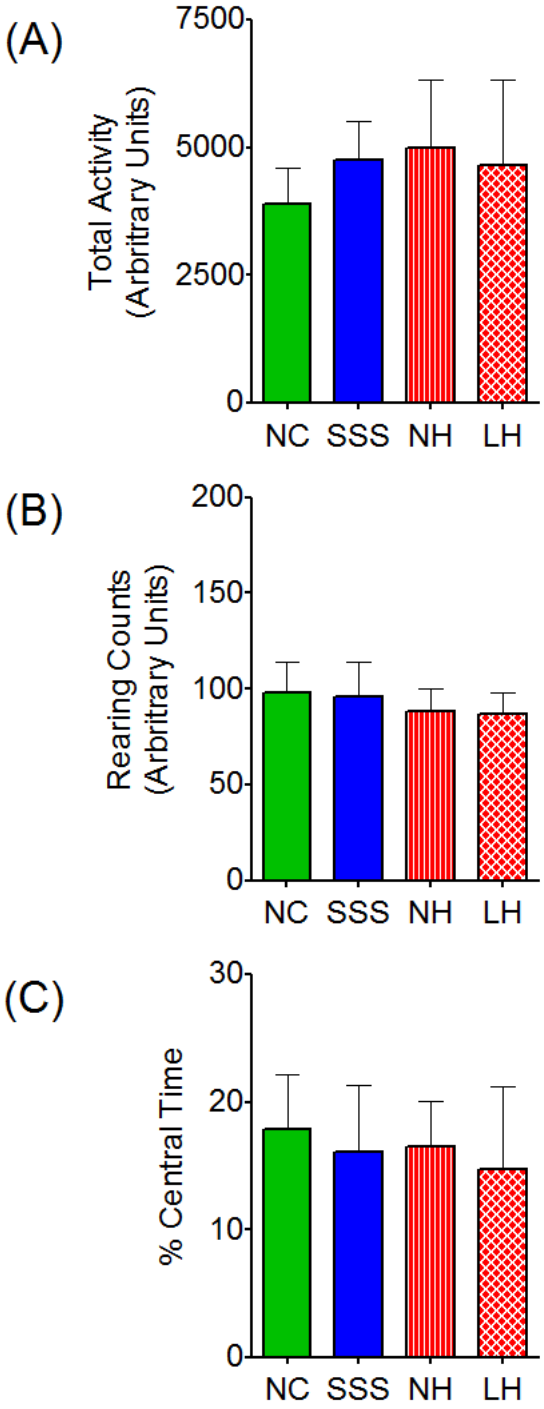
(A)
Total Activity
(Arbitrary Units)
7500
5000
2500
0
NC
SSS
NH
LH
(B)
Rearing Counts
(Arbitrary Units)
200
150
100
50
0
NC
SSS
NH
LH
(C)
% Central Time
30
20
10
0
NC
SSS
NH
LH

Figure 4: Locomotor activity and exploratory activity and anxiety behavior did not differ between groups in the Open Field Test between the NC, SSS, LH and NH rats as determined by previous cluster analysis.
One day after the escape behavior testing, NC, SSS, LH and NH rats were subjected to the open field test to assess locomotor and exploratory activities as well as to assess anxiety.
Panel A - Exploratory activity as indicated by total activity.
Panel B - Locomotor activity as indicated by rearing counts.
Panel C - Anxiety behavior as indicated by % central time. Data are presented as mean±S.E.M. No significant differences were found.

4.1.3. Elevated Plus Maze test (EPM)

All rats from the non-swim control (n=10), single sham swim control (n=10) and single forced swim stress (n=10) groups were subjected to the elevated plus maze test. This test is considered a more reliable indicator of anxiety than the open field test. The elevated plus maze employs height-induced fear in rats and their proclivity to remain in the closed area as opposed to open areas as indices of anxiety. A decrease in open arm activity (duration and/or entries) is considered as indicative of increased anxiety-like behavior. There were no differences between non-swim control, single sham swim control, NH and LH rats, in any of the anxiety parameters assessed in EPM (Fig 5).

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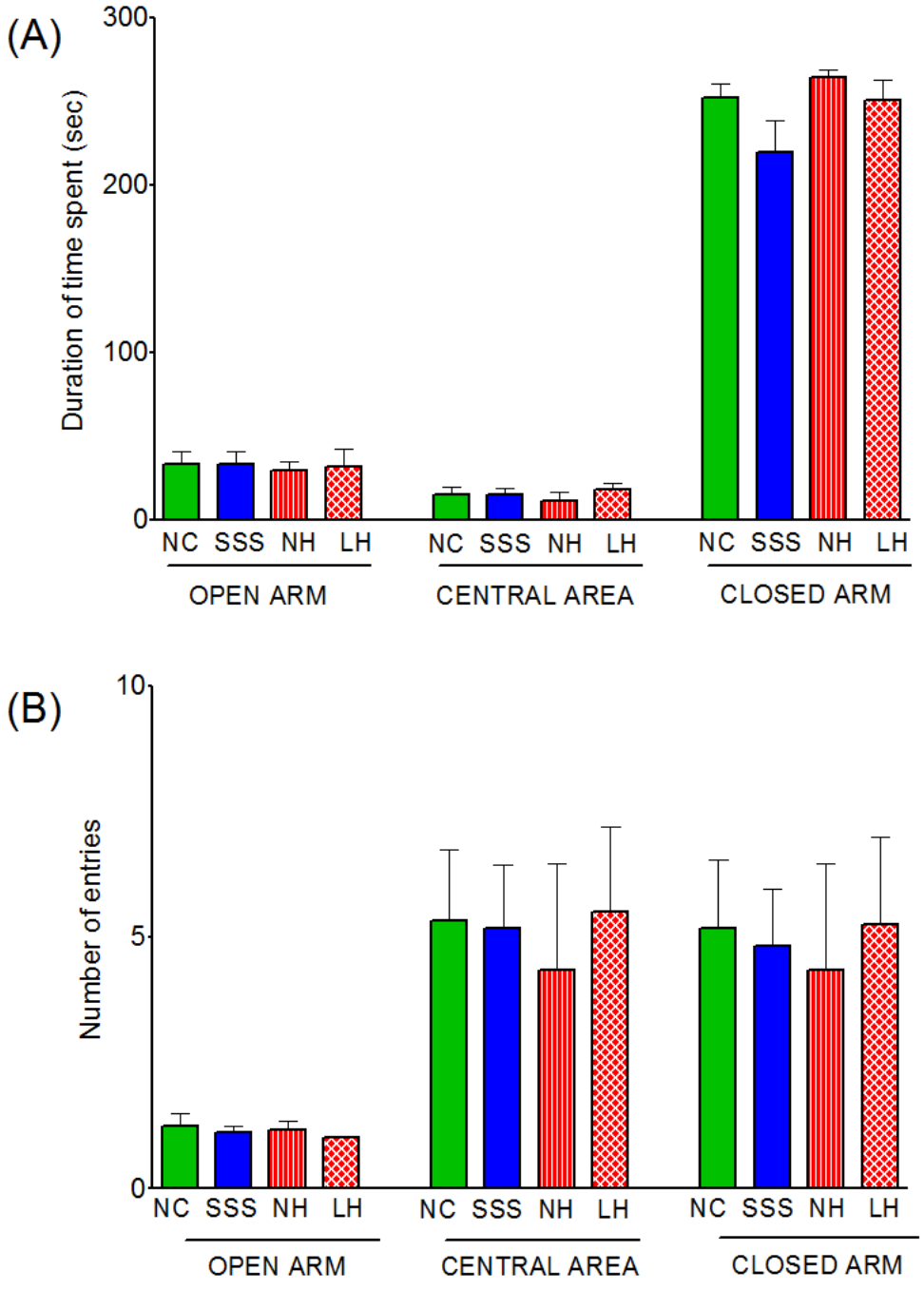

(A)
Duration of time spent (sec)
300
200
100
0
NC SSS NH LH
NC SSS NH LH
NC SSS NH LH
OPEN ARM
CENTRAL AREA
CLOSED ARM
(B)
Number of entries
10
5
0
NC SSS NH LH
NC SSS NH LH
NC SSS NH LH
OPEN ARM
CENTRAL AREA
CLOSED ARM

Figure 5: No difference in the Elevated Plus Maze parameters between the NC, SSS, LH and NH rats, from single forced swim stress group.
One day after the shuttle-box, the NC (n=10), SSS (n=10), LH (n=4) and NH (n=6) rats were subjected to the elevated plus maze to assess anxiety.
Panel A – Duration of time spent by the animals in open arm, central area and the closed arm.
Panel B - Number of entries undertaken by the animals into the open arm, central area and the closed arm. The performance of all rats was similar in all the parameters investigated. Data are presented as mean±S.E.M. No significant differences were found.

4.1.4. Effects of single forced swim stress on levels of GRK3/2, TH, α_{2A}-AR and CRF_1-R in LC of rats.

There were no changes in the levels of GRK3 (Fig 6A), GRK2 (Fig 7A), TH (Fig 8A), α_{2A}-AR (Fig 9A) and CRF_1-R (Fig 10A) in LC of rats exposed to a SFS compared to the NC and SSS, determined 48h after exposure to a single forced swim stress. When the levels of GRK3, GRK2, TH, α_{2A}-AR and CRF_1-R in LC of LH and NH rats, the groups found from cluster analysis of the SFS, were compare, no significant differences were observed between them and neither group demonstrated any significant difference from the non-swim or single sham swim controls (Fig 6B, 7B, 8B, 9B and 10B). Thus, exposure to a single forced swim stress did not produce any changes in the levels of GRK3, GRK2, TH, α_{2A}-AR and CRF_1-R in the LC of rats 48 h after the stress.

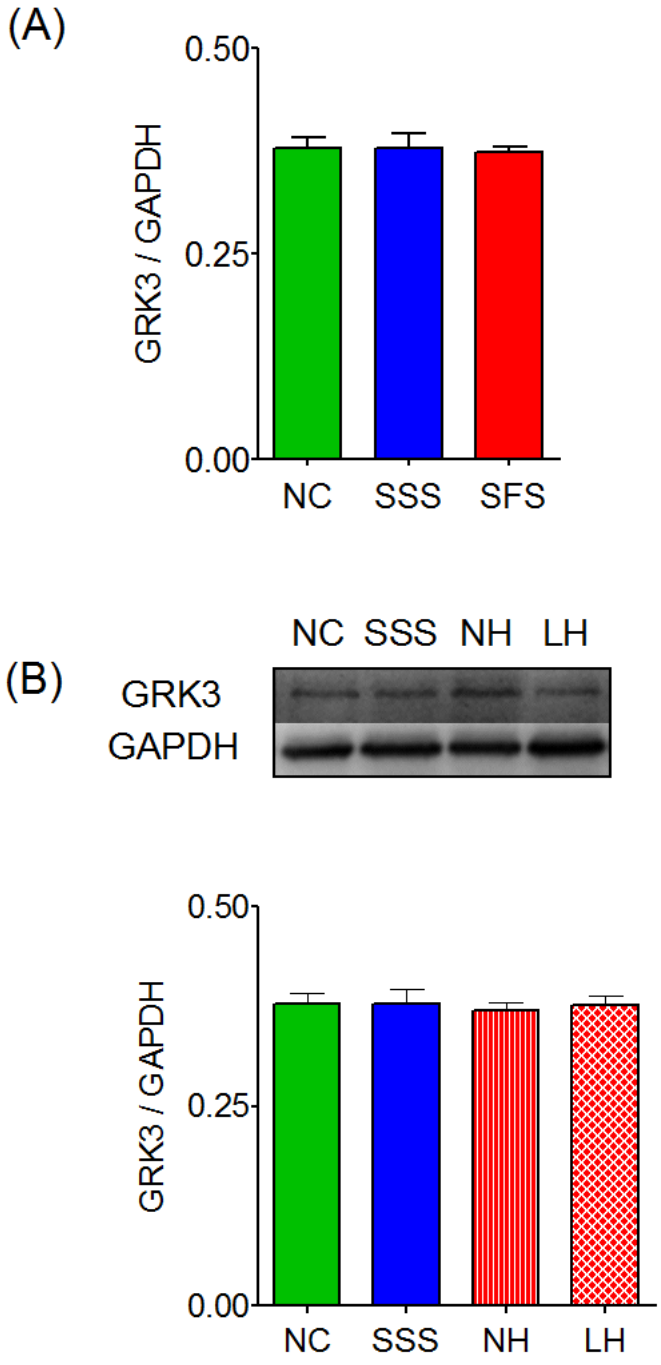

(A)
0.50
0.25
0.00
GRK3 / GAPDH
NC
SSS
SFS
(B)
NC SSS NH LH
GRK3
GAPDH
0.50
0.25
0.00
GRK3 / GAPDH
NC
SSS
NH
LH

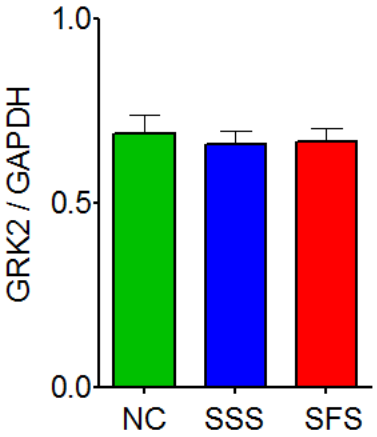
Figure 6: No change in levels of GRK3 in LC of rats exposed to a single forced swim stress.

Fourty-eight hours after the swim stress, brains were harvested and levels of GRK3 were determined by western blot analysis.

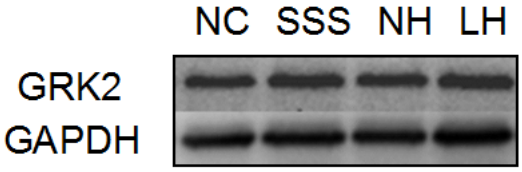
Panel A – Mean levels of GRK3 in the LC of the NC (n=12), SSS (n=10) and SFS (n=10) groups.

Panel B – Representative western blots and mean levels of GRK3 in NC (n=12), SSS (n=10), LH (n=4) and NH (n=6) groups. Protein levels are expressed as ratio of intensity of the immunoreactive band for GRK3 normalized by the band for the loading control, GAPDH, and presented as mean±S.E.M. No significant differences were observed between the groups.

(A)


1.0
0.5
0.0
GRK2 / GAPDH
NC
SSS
SFS

(B)


NC SSS NH LH
GRK2
GAPDH

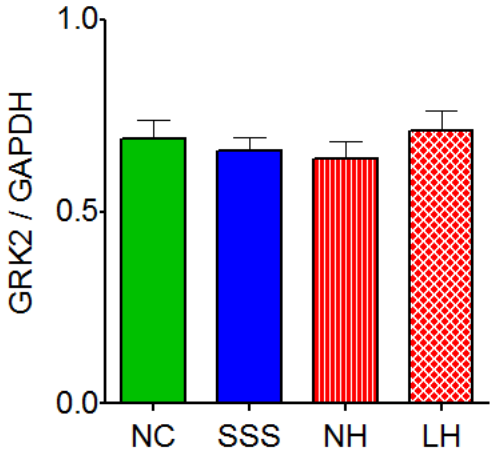
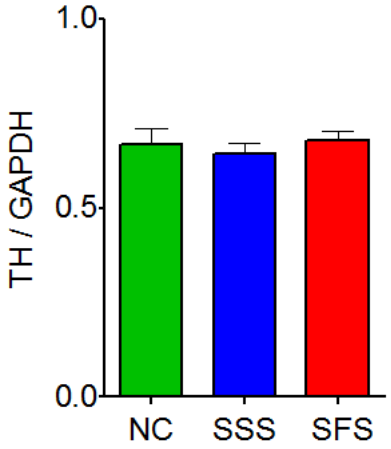
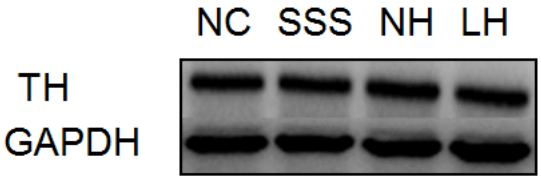

1.0
0.5
0.0
GRK2 / GAPDH
NC
SSS
NH
LH

Figure 7: No change in levels of GRK2 in LC of rats exposed to single forced swim stress.
Fourty-eight hours after the swim stress brains were harvested and levels of GRK2 were determined by western Blot analysis.
Panel A – Mean levels of GRK2 in the LC of the NC (n=12), SSS (n=10) and SFS (n=10) groups.
Panel B – Representative western blots and mean levels of GRK2 in NC (n=12), SSS (n=10), LH (n=4) and NH (n=6) groups. Protein levels are expressed as ratio of intensity of the immunoreactive band for GRK2 normalized by the band for the loading control, GAPDH, and presented as mean±S.E.M. No significant differences were observed between the groups.

(A)


1.0
0.5
0.0
TH / GAPDH
NC
SSS
SFS

(B)


NC
SSS
NH
LH
TH
GAPDH

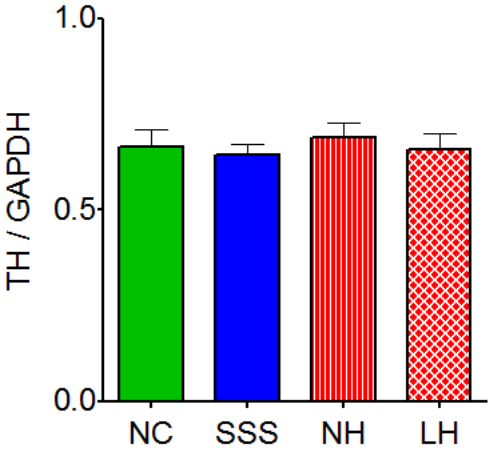

1.0
0.5
0.0
TH / GAPDH
NC
SSS
NH
LH

Figure 8: No change in levels of TH in LC of rats exposed to single forced swim stress.

Fourty-eight hours after the swim stress, brains were harvested and levels of TH were determined by western Blot analysis.

Panel A – Mean levels of TH in the LC of the NC (n=12), SSS (n=10) and SFS (n=10) groups.

Panel B – Representative western blots and mean levels of TH in NC (n=12), SSS (n=10), LH (n=4) and NH (n=6) groups. Protein levels are expressed as ratio of intensity of the immunoreactive band for TH normalized by the band for the loading control, GAPDH, and presented as mean±S.E.M. No significant differences were observed between the groups.

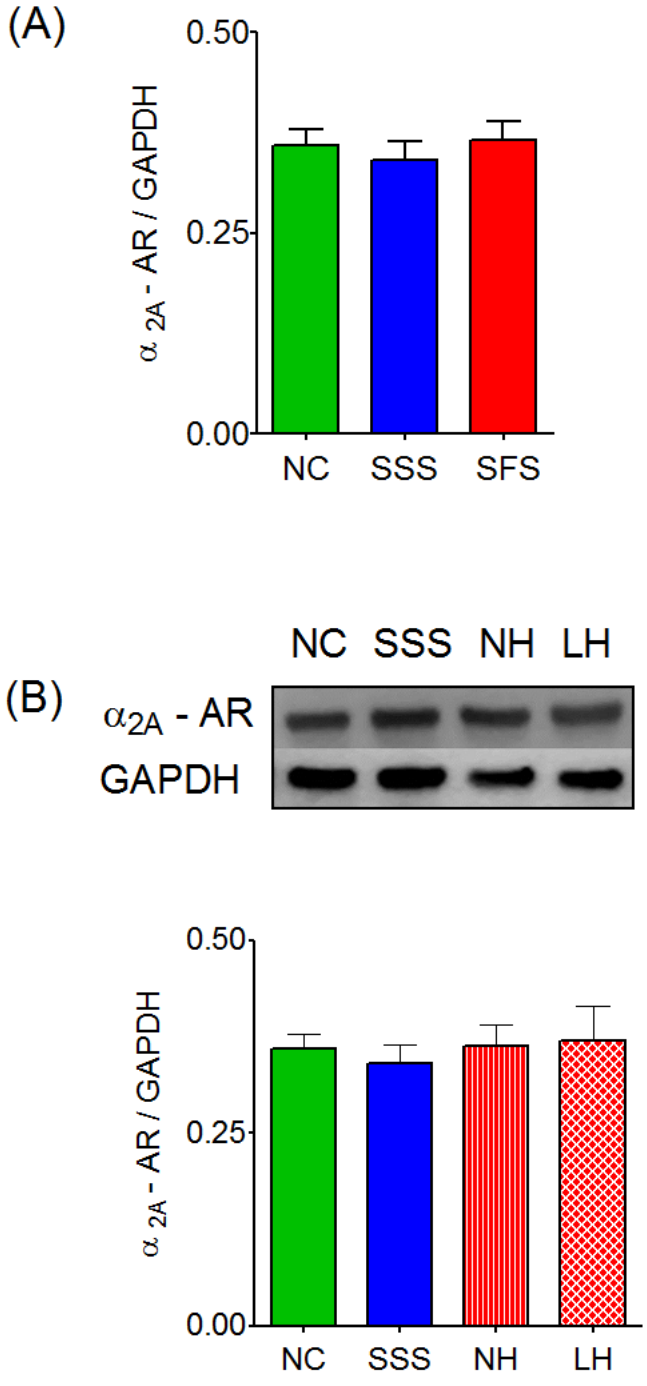

(A)
0.50
0.25
0.00
α 2A - AR / GAPDH
NC
SSS
SFS
(B)
NC SSS NH LH
α2A - AR
GAPDH
0.50
0.25
0.00
α 2A - AR / GAPDH
NC
SSS
NH
LH

Figure 9: No change in levels of α_{2A}-AR in LC of rats exposed to single forced swim stress.

Fourty-eight hours after the swim stress, brains were harvested and levels of α_{2A}-AR were determined by western Blot analysis.

Panel A – Mean levels of α_{2A}-AR in the LC of NC (n=12), SSS (n=10) and SFS (n=10) groups.

Panel B – Representative western blots and mean levels of α_{2A}-AR in NC (n=12), SSS (n=10), LH (n=4) and NH (n=6) groups. Protein levels are expressed as ratio of intensity of the immunoreactive band for α_{2A}-AR normalized by the band for the loading control, GAPDH, and presented as mean±S.E.M. No significant differences were observed between the groups.

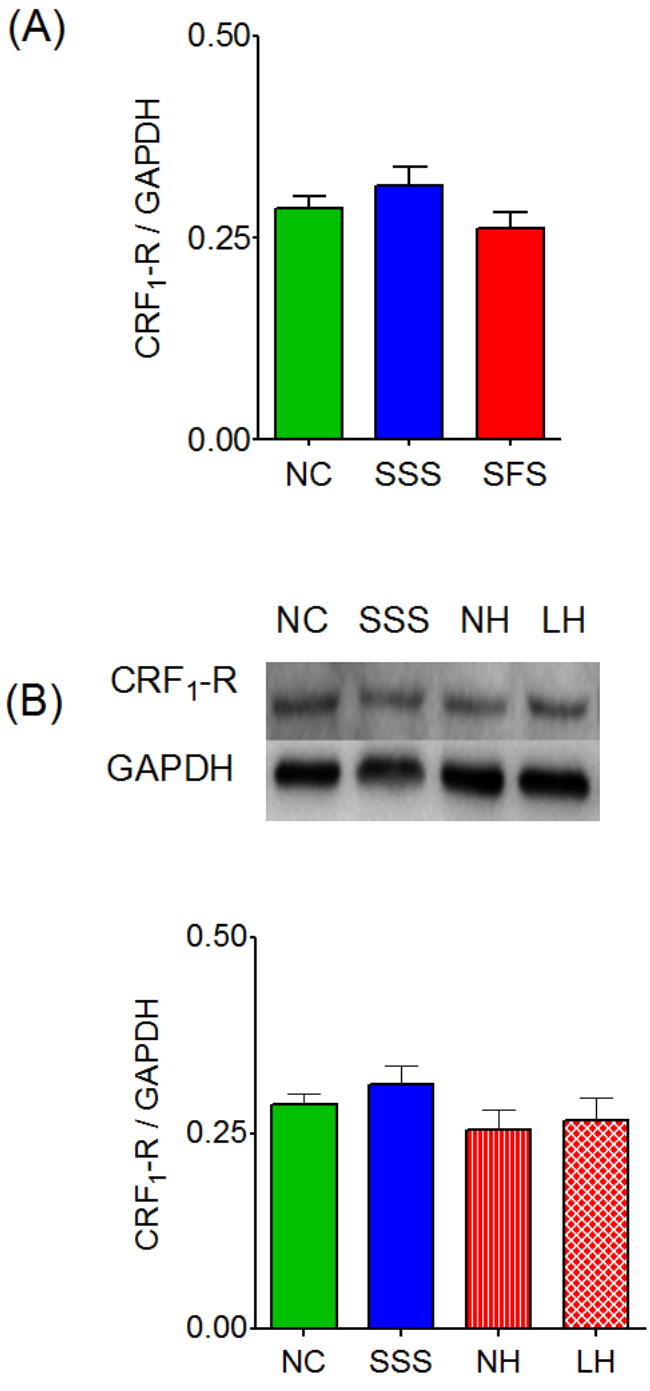

(A)
0.50
0.25
0.00
CRF1-R / GAPDH
NC
SSS
SFS
(B)
NC
SSS
NH
LH
CRF1-R
GAPDH
0.50
0.25
0.00
CRF1-R / GAPDH
NC
SSS
NH
LH

Figure 10: No change in levels of CRF_1-R in LC of rats exposed to single forced swim stress.

Fourty-eight hours after the swim stress, brains were harvested and levels of CRF_1-R were determined by western Blot analysis.

Panel A – Mean levels of CRF_1-R in the LC of the normal control NC (n=12), SSS (n=10) and SFS (n=10) groups.

Panel B – Representative western blots and mean levels of CRF_1-R in NC (n=12), SSS (n=10), LH (n=4) and NH (n=6) groups. Protein levels are expressed as ratio of intensity of the immunoreactive band for CRF_1-R normalized by the band for the loading control, GAPDH, and presented as mean±S.E.M. No significant differences were observed between the groups.

4.2. Repeated forced swim stress – 3 daily sessions.

4.2.1. Three daily sessions of forced swim stress increases immobility during the swim stress.

Rats were subjected to repeated forced swim for three days. The behavioral parameters analyzed during the swim stress were swimming and climbing (active behaviors arising from attempts made by the rats to escape from the situation) and immobility (passive behavior which reveals a despair condition). Repeated forced swim stress caused an increase in immobility counts during the first 10 min of day 3 as compared to day 1 or day 2, $p<0.05$ (Fig 11A). Also, there was decreased swimming behavior decreased during the first 10 min of day 3 compared to day 1 or day 2, $p<0.05$ (Fig 11C). However, there was no change in climbing behavior between any of the days (Fig 11B). Thus, repeated forced swim stress significantly increased immobility, an index of behavioral despair.

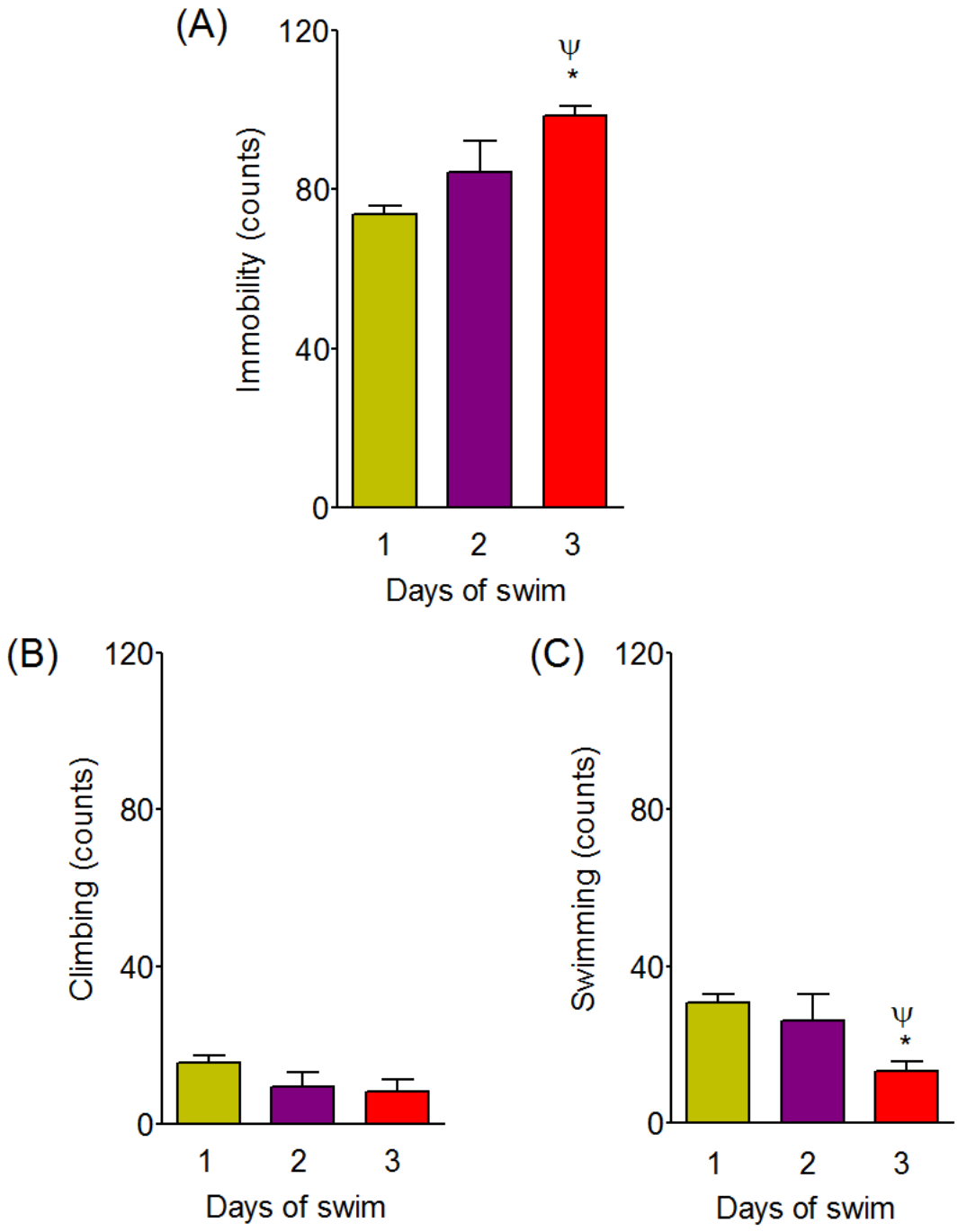

(A)
120
80
40
0
Immobility (counts)
ψ
*
1
2
3
Days of swim
(B)
120
80
40
0
Climbing (counts)
1
2
3
Days of swim
(C)
120
80
40
0
Swimming (counts)
ψ
*
1
2
3
Days of swim

Figure 11: Repeated forced swim stress for three days increased immobility.

Rats were subjected to repeated forced swim stress for three days by exposing them to 30 cm deep water for 10 min on day 1 and 20 min each on day 2 and day 3. The behavioral parameters analyzed were swimming and climbing and immobility. The predominant behavior (swimming, climbing or immobility) observed during each 5 sec period was scored by a blinded observer. The total number of counts for each behavioral that was scored, in the first 10 min of swim, was noted. Mean count for each group for each of three days was determined.

Panel A - Immobility counts on days 1, 2 and 3 of forced swim stress.

Panel B - Climbing counts on days 1, 2 and 3 of stress.

Panel C - Swimming counts on days 1, 2 and 3 of stress. The data are presented as mean±S.E.M. of the number of counts for each behavior on each day and * and Ψ indicate significantly different from day 1 and day 2, respectively, $p<0.05$.

4.2.2. Repeated forced swim stress for 3 days changed escape behavior in the shuttle-box.

The rats were subjected to repeated forced swim stress for three days. Then, 24 hr after the last swim, rats were tested for escape behavior in the shuttle-box. In the 5 fixed ratio 1 (FR-1) trials, used to rule out any motor deficit in the rats, no differences between any of the groups, non-swim control (NC), repeated sham swim control (RSS) and repeated forced swim stress (RFS) groups were noted (Fig 12 A, B). This ruled out any differences in locomotor function between the groups.

When they were subjected to the fixed ratio 2 (FR2) trials, the RFS rats did not demonstrate any significant difference in the mean escape latency when compared to either the non-swim control or the repeated sham swim control group (Fig 13 A). However, the repeated forced swim stress rats demonstrated a significant difference in the percentage escape failure when compared to either the non-swim control or the repeated sham swim control rats (Fig 13B). When K-means cluster analysis was applied to the behavior data (mean escape latency and percentage escape failure) from the repeated forced swim stress group, the analysis bifurcated the data into two clusters (Fig 13C, D). These clusters significantly differed from each other. One of these clusters showed significantly impaired escape behavior (significantly increased mean escape latency and

significantly increased percentage escape failure) compared to either the non-swim control or repeated sham swim control and was termed as Learned Helpless (LH). The other cluster was not significantly different from the controls and was termed as Non Helpless (NH).

The FR1 data of the LH and NH rats were compared with the controls (Fig 12 C, D), but were not found to differ significantly. This indicated that in the FR2 trials, any differences between the groups were not due to locomotor deficits within the clusters of the stressed rats. This rule out motor deficits as potential causes for differences that might be observed between the NH and LH clusters in the FR2 trials.

FR1

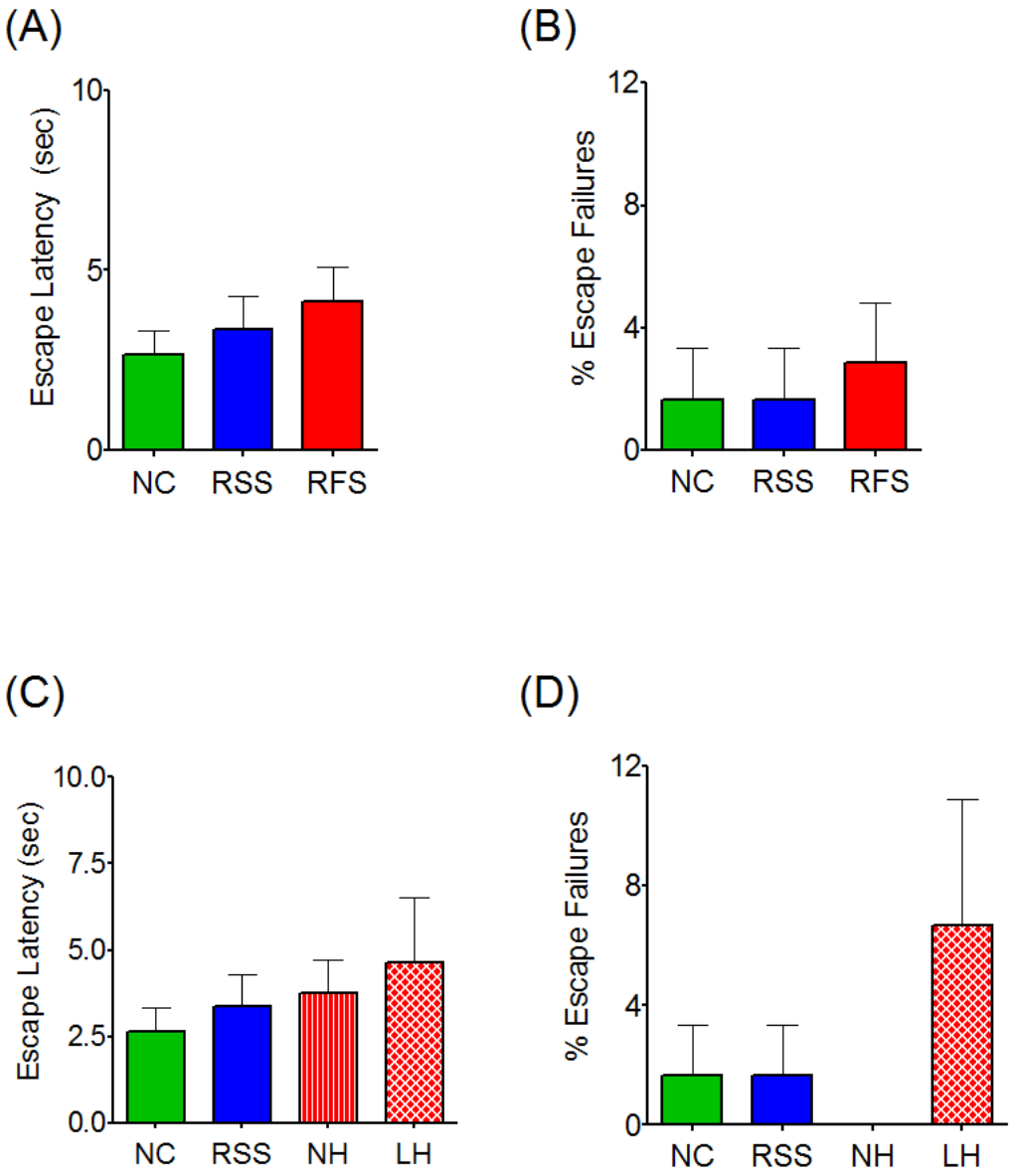

(A)
Escape Latency (sec)
10
5
0
NC
RSS
RFS
(B)
% Escape Failures
12
8
4
0
NC
RSS
RFS
(C)
Escape Latency (sec)
10.0
7.5
5.0
2.5
0.0
NC
RSS
NH
LH
(D)
% Escape Failures
12
8
4
0
NC
RSS
NH
LH

Figure 12: Exposure of rats to repeated forced swim stress for three days did not demonstrate any motor deficits in the fixed ratio 1 (FR1) trials.
Rats were randomly assigned to three groups, named non-swim control (NC, n=12), repeated sham swim control (RSS, n=12) and repeated forced swim stress (RFS, n=12) as described above. Twenty-four hours after the last swim all the rats were tested for escape behavior in the shuttle-box. First the rats were observed for any motor deficits using 5 fixed ratio 1 (FR1) trials. The FR1 data of the Learned Helpless (LH) and the Non Helpless (NH) rats as mentioned above were also compared with the controls (NC and RSS). No escape failures were observed in the groups where there are no bars.
Panel A – Mean escape latencies for NC, RSS and RFS rats for the FR1 trials. **Panel B –** Percentage escape failures for NC, RSS and RFS for the FR1 trials. **Panel C and D –** Mean escape latencies and percentage escape failures for LH (n=6) and NH (n=6) rats for the FR1 trials. Data are presented as mean±S.E.M.

FR2

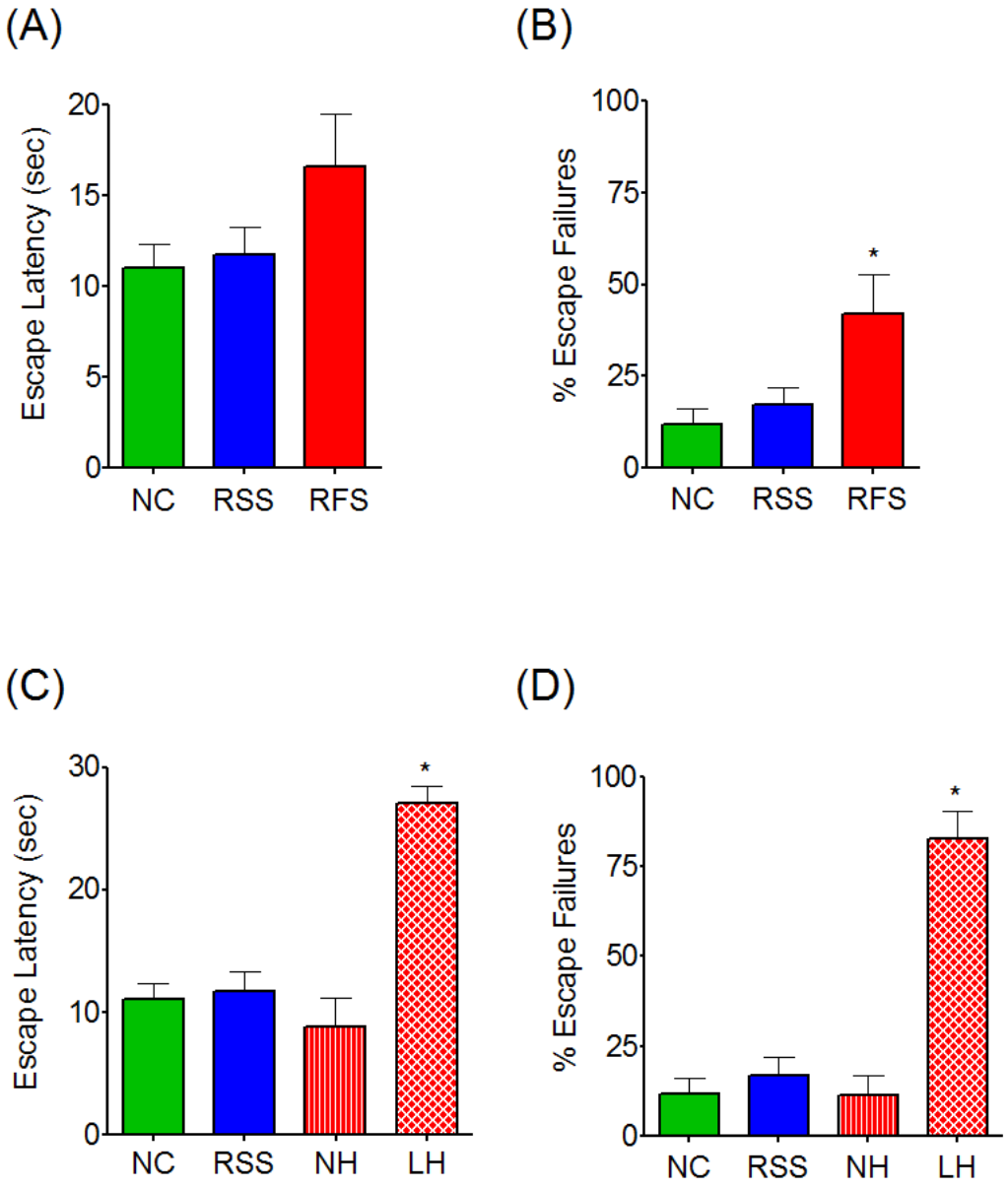

(A)
Escape Latency (sec)
20
15
10
5
0
NC
RSS
RFS
(B)
% Escape Failures
100
75
50
25
0
*
NC
RSS
RFS
(C)
Escape Latency (sec)
30
20
10
0
*
NC
RSS
NH
LH
(D)
% Escape Failures
100
75
50
25
0
*
NC
RSS
NH
LH

Figure 13: Exposure of rats to repeated forced swim stress for three days resulted in two behaviorally different groups in FR2 trials.
Rats were randomly assigned to three groups, named NC (n=12), RSS (n=10) and RFS (n=10) as described above. Twenty-four hours after the last swim all the rats were tested for escape behavior in the shuttle-box subjecting each rat to 25 fixed ratio 2 (FR2) trials to observe the escape behavior as described above.
Panel (A) – Mean escape latencies for NC, RSS and RFS rats for the FR2 trials.
Panel (B) – Percentage escape failures for NC, RSS and RFS for the FR2 trials.
Panel (C and D) – Mean escape latencies and percentage escape failures for LH (n=6) and NH (n=6) rats for the FR2 trials, as identified from k-means cluster analysis. Data are presented as mean±S.E.M. and * indicates significantly different from NC, RSS, and NH groups, $p<0.05$.

4.2.3. Open field test

The open field test allowed investigation of the locomotor and exploratory behavior of the non-swim control, repeated sham swim control and repeated forced swim stressed LH and NH rats. There were no significant differences in the locomotor activity, assessed by total activity, between any of the groups (Fig 14A). Also, exploratory behavior of the rats, assessed by their rearing counts (Fig 14B), did not demonstrate any significant differences between NC, RSS, NH and LH rats. Anxiety behavior also was evaluated from the open field test. This was done by calculating the percentage of total time spent by rats in the central compartment. No significant differences amongst NC, RSS, NH and LH rats were noted for this parameter (Fig 14C).

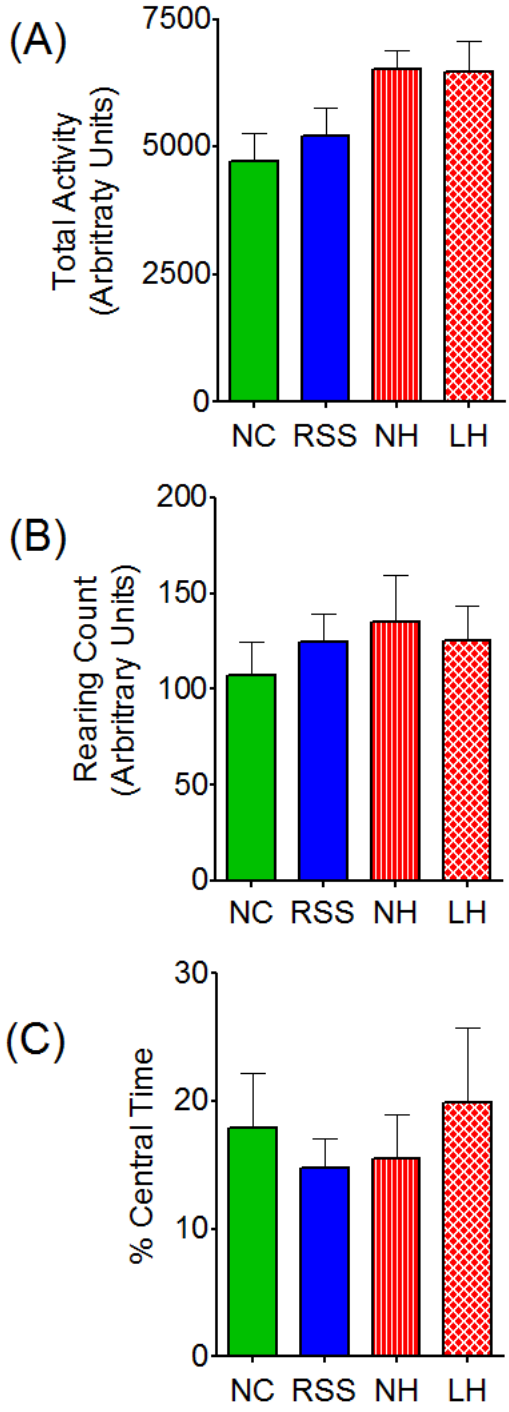

(A)
Total Activity
(Arbitraty Units)
7500
5000
2500
0
NC RSS NH LH
(B)
Rearing Count
(Arbritrary Units)
200
150
100
50
0
NC RSS NH LH
(C)
% Central Time
30
20
10
0
NC RSS NH LH

Figure 14: No differences in locomotor activity or exploratory activity or anxiety behavior in the Open Field Test between the NC, RSS, LH and NH groups.

One day after the escape testing NC, RSS, LH and NH rats were subjected to the open field test to assess locomotor and exploratory activities as well as to assess anxiety.

Panel A - Exploratory activity as indicated by total activity.

Panel B - Locomotor activity as indicated by rearing counts.

Panel C - Anxiety behavior as indicated by % central time. Data are presented as mean±S.E.M. No significant differences were found, $p<0.05$.

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4.2.4. Elevated plus Maze test

All rats were subjected to the elevated plus maze test. This test is considered a more reliable indicator of anxiety than open field test. The elevated plus maze employs height-induced fear in rats and a rat's proclivity to remain in the closed area as opposed to open areas as indices of anxiety. A decrease in open arm activity (duration and/or entries) is considered indicative of increased anxiety-like behavior. No differences between non-swim control, repeated sham swim control, LH and NH rats were observed in the anxiety parameters (Fig 15).

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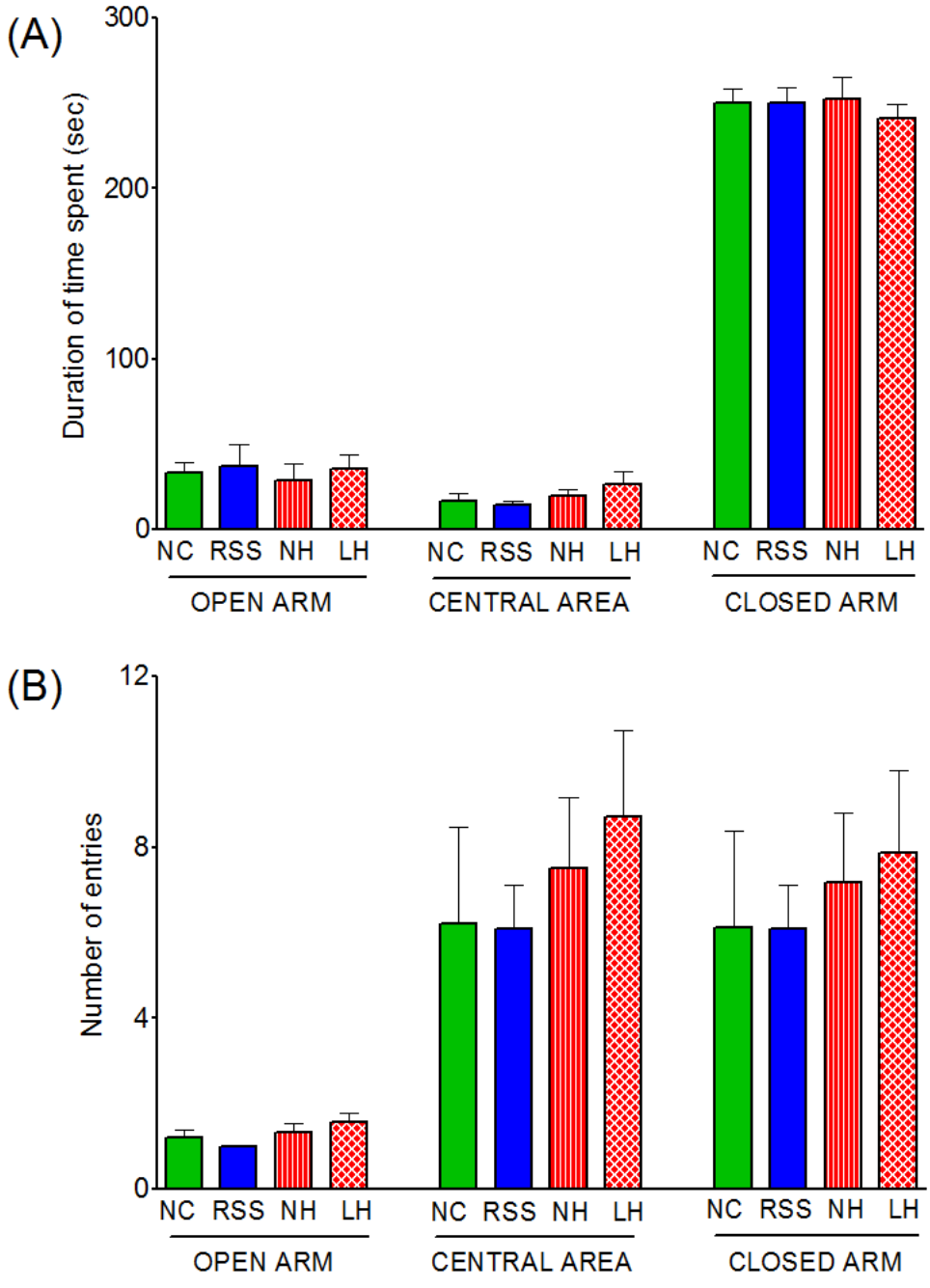

(A)
Duration of time spent (sec)
300
200
100
0
NC RSS NH LH
NC RSS NH LH
NC RSS NH LH
OPEN ARM
CENTRAL AREA
CLOSED ARM
(B)
Number of entries
12
8
4
0
NC RSS NH LH
NC RSS NH LH
NC RSS NH LH
OPEN ARM
CENTRAL AREA
CLOSED ARM

Figure 15: No difference in the Elevated Plus Maze parameters between the NC, RSS, LH and NH groups.

One day after the shuttle-box, the NC (n=12), RSS (n=12), LH (n=6) and NH (n=6) rats were subjected to the elevated plus maze to assess anxiety.

Panel A – Duration of time spent by the animals in open arm, central area and the closed arm.

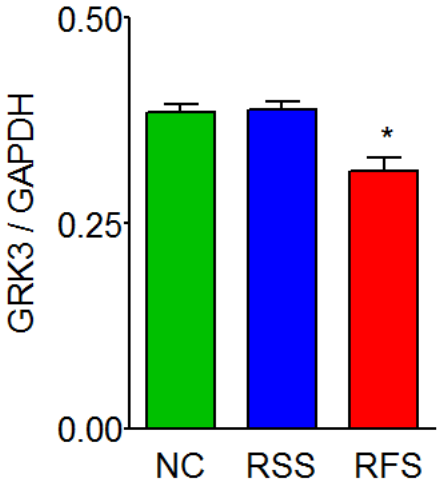
Panel B - Number of entries undertaken by the animals into the open arm, central area and the closed arm. The performance of all rats was similar in all the parameters investigated. Data are presented as mean±S.E.M. No significant differences were found.

4.2.5. Effects of repeated forced swim stress on levels of GRK3/2, TH, α_{2A}-AR and CRF_1-R in LC of rats.

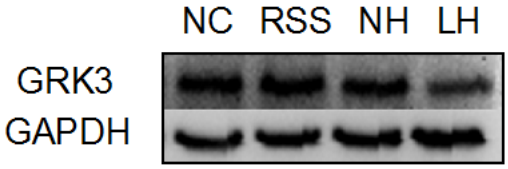
Unlike rats subjected to a single forced swim stress, the levels of GRK3 (17.8%, $p<0.05$) in the LC of rats exposed to repeated forced swim stress (RFS) rats were decreased compared to the non-swim control (NC) and repeated sham swim control (RSS) (Fig 16A). Also levels of TH (21.3%, $p<0.05$) were increased in the LC of rats exposed to repeated forced swim stress compared to the NC and RSS (Fig 18A). There were no changes in levels of GRK2 (Fig 17A), α_{2A}-AR (Fig 19A) and CRF_1-R (Fig 20A) in LC of rats exposed to repeated forced swim stress compared to the non-swim and repeated sham swim controls. Levels of GRK3, GRK2, TH, α_{2A}-AR and CRF_1-R in LC of LH and NH clusters, identified from the escape testing of stressed rats, also were compared. There were significant decreases in the levels of GRK3 in LC of the LH rats compared to NC (28.4%. $p<0.05$), repeated sham swim control and the NH rats (Fig 16B). No significant differences were observed in the levels of GRK2 in LC of rats in the NC, RSS, LH and NH groups (Fig 17B). Levels of TH in the LC of the LH rats showed significant increases as compared to the NC (35.5%, $p<0.05$), RSS or NH (Fig 18B). Though it was not apparent when comparing stressed to unstressed rats, there also was a significant increase in the levels of α_{2A}-AR in LC of LH rats compared to NC (33.53%, $p<0.05$), RSS or NH groups (Fig 19B).

Similarly, the levels of CRF_1-R in LC demonstrated a significant increase in the LH rats when compared to the NC (29.4%, $p<0.05$), RSS or NH (Fig 20B). Thus there were behaviorally associated changes in the levels of GRK3, TH, α_{2A}-AR and CRF_1-R in LC of rats exposed to repeated forced swim stress for three days.

(A)


0.50
0.25
0.00
GRK3 / GAPDH
*
NC
RSS
RFS

(B)


NC
RSS
NH
LH
GRK3
GAPDH

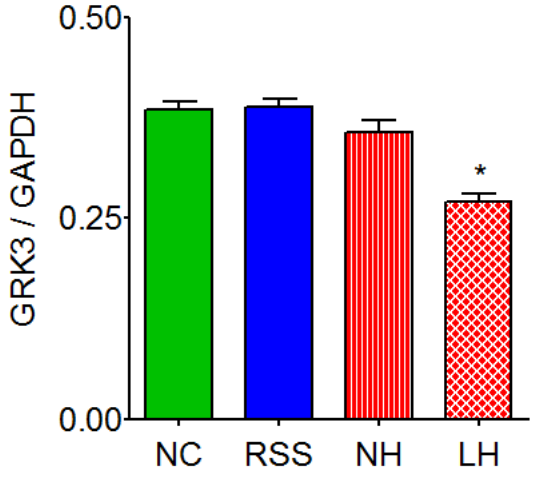
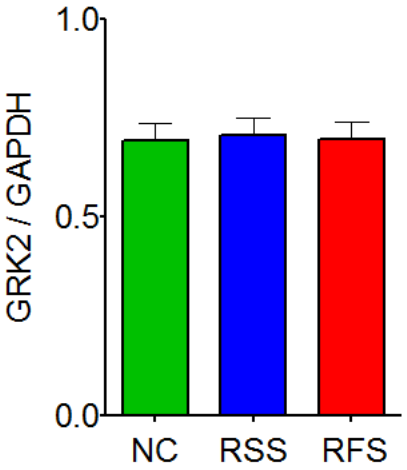
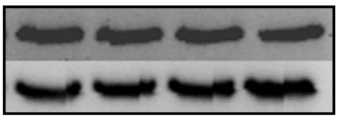

0.50
0.25
0.00
GRK3 / GAPDH
*
NC
RSS
NH
LH

Figure 16: GRK3 levels are decreased in LC of rats made LH by RFS.
Forty-eight hours after the swim stress, brains were harvested and levels of GRK3 were determined by western blot analysis.
Panel A – Mean levels of GRK3 in the LC of the NC (n=12), RSS (n=12) and RFS (n=12) groups. There was a decrease (17.8%, $p<0.05$) in the levels of GRK3 in LC of RFS rats compared to the NC. Data are presented as mean±S.E.M. and * indicates significantly different from NC and RSS groups, $p<0.05$.
Panel B – Representative western blots and mean levels of GRK3 in NC, RSS, LH (n=6) and NH (n=6) groups. There was a decrease (28.3%, $p<0.05$) in the levels of GRK3 in LC of LH rats compared to the NC. Protein levels are expressed as ratio of intensity of the immunoreactive band for GRK3 normalized by the band for the loading control, GAPDH, and presented as mean±S.E.M. and * indicates significantly different from NC, RSS, and NH groups, $p<0.05$.

(A)



(B)



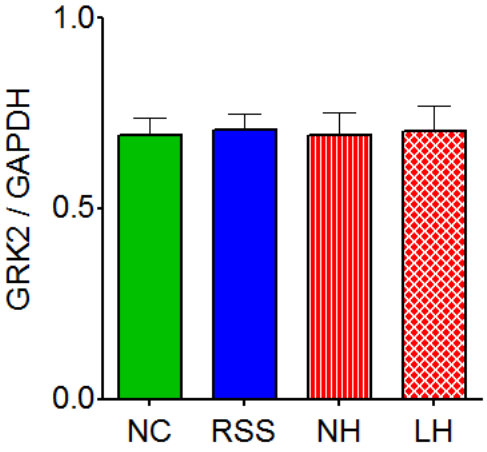
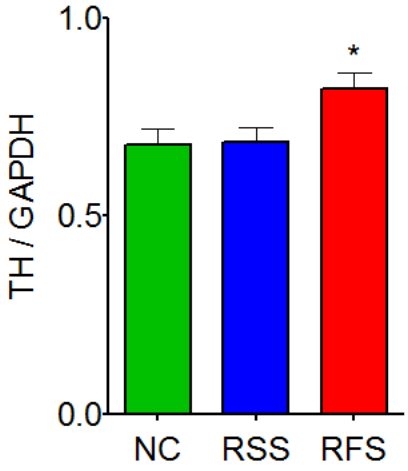
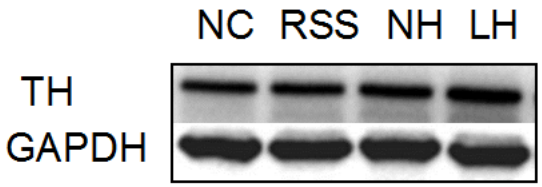


Figure 17: GRK2 levels did not change in LC of rats made LH by RFS.
Forty-eight hours after the swim stress, brains were harvested and levels of GRK2 were determined by western blot analysis.
Panel A – Mean levels of GRK2 in the LC of the normal control (NC, n=12), repeated sham swim control (RSS, n=12) and repeated forced swim stress (RFS, n=12) groups. Data are presented as mean±S.E.M.
Panel B – Representative western blots and mean levels of GRK2 in NC, RSS, Learned Helpless (LH, n=6) and Non Helpless (NH, n=6) groups. Protein levels are expressed as ratio of intensity of the immunoreactive band for GRK2 normalized by the band for the loading control, GAPDH, and presented as mean±S.E.M. No significant differences were observed between the groups.

(A)


1.0
0.5
0.0
TH / GAPDH
*
NC
RSS
RFS

(B)


NC RSS NH LH
TH
GAPDH

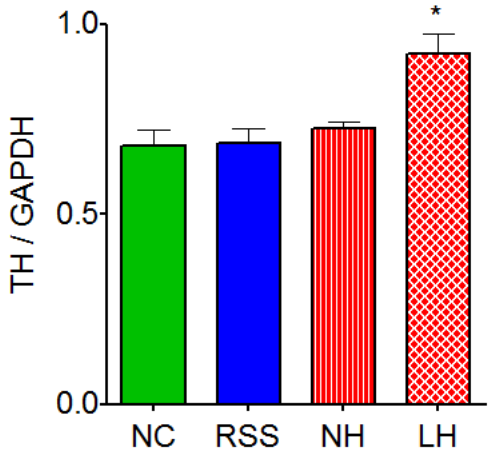
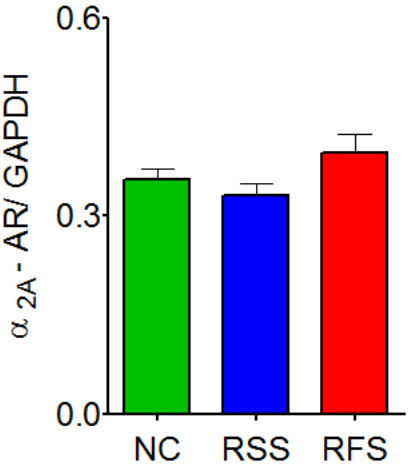
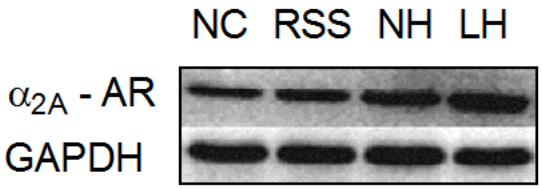

1.0
0.5
0.0
TH / GAPDH
*
NC
RSS
NH
LH

Figure 18: TH levels are increased in LC of rats made LH by RFS.
Forty-eight hours after the swim stress, brains were harvested and levels of GRK3 were determined by western blot analysis.
Panel A – Mean levels of TH in the LC of the NC (n=12), RSS (n=12) and RFS (n=12) groups. There was a increase (21.3%, $p<0.05$) in the levels of TH in LC of RFS rats compared to the NC. Data are presented as mean±S.E.M. and * indicates significantly different from NC and RSS groups, $p<0.05$.
Panel B – Representative western blots and mean levels of TH in NC, RSS, LH (n=6) and NH (n=6) groups. There was an increase (35.5%, $p<0.05$) in the levels of TH in LC of LH rats compared to the NC. Protein levels are expressed as ratio of intensity of the immunoreactive band for TH normalized by the band for the loading control, GAPDH, and presented as mean±S.E.M. and * indicates significantly different from NC, RSS, and NH groups, $p<0.05$.

(A)


0.6
0.3
0.0
α 2A - AR/ GAPDH
NC
RSS
RFS

(B)


NC RSS NH LH
α2A - AR
GAPDH

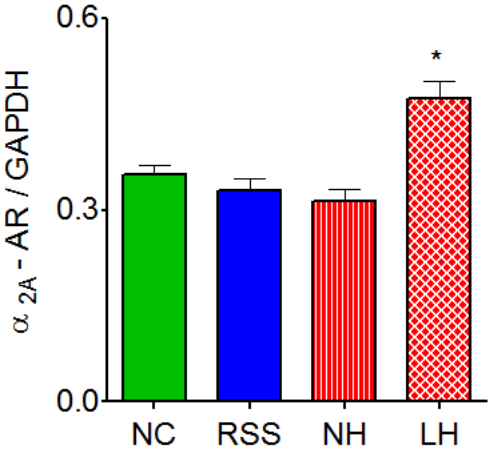
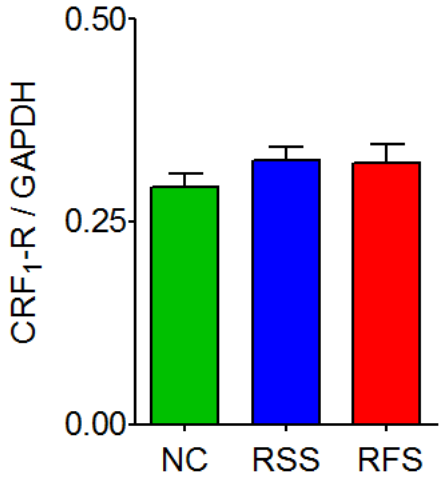
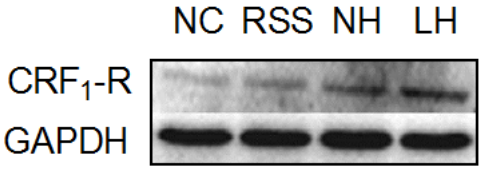

0.6
0.3
0.0
α 2A - AR / GAPDH
*
NC
RSS
NH
LH

Figure 19: **α_{2A}-AR levels are increased in LC of rats made LH by RFS.**
Forty-eight hours after the swim stress, brains were harvested and levels of α_{2A}-AR were determined by western blot analysis.
Panel A – Mean levels of α_{2A}-AR in the LC of the NC (n=12), RSS (n=12) and RFS (n=12) groups. There was no change in the levels of α_{2A}-AR in LC of RFS rats compared to the NC or RSS. Data are presented as mean±S.E.M
Panel B – Representative western blots and mean levels of α_{2A}-AR in NC, RSS, LH (n=6) and NH (n=6) groups. There was an increase (32.6 %, $p<0.05$) in the levels of α_{2A}-AR in LC of LH rats as compared to the NC. Protein levels are expressed as ratio of intensity of the immunoreactive band for α_{2A}-AR normalized by the band for the loading control, GAPDH, and presented as mean±S.E.M. and * indicates significantly different from NC, RSS, and NH groups, $p<0.05$.

(A)


0.50
0.25
0.00
CRF1-R / GAPDH
NC
RSS
RFS

(B)


NC RSS NH LH
CRF1-R
GAPDH

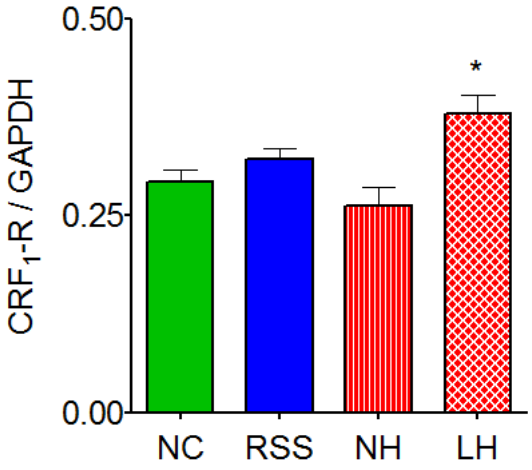

0.50
0.25
0.00
CRF1-R / GAPDH
*
NC
RSS
NH
LH

Figure 20: CRF_1-R levels are increased in LC of made LH by RFS.
Forty-eight hours after the swim stress, brains were harvested and levels CRF_1-R were determined by western blot analysis.
Panel A – Mean levels of CRF_1-R in the LC of the NC (n=12), RSS (n=12) and RFS (n=12) groups. There was no change in the levels of CRF_1-R in LC of RFS rats compared to the NC or RSS. Data are presented as mean±S.E.M
Panel B – Representative western blots and mean levels of CRF_1-R in NC, RSS, LH (n=6) and NH (n=6) groups. There was an increase (29.4 %, $p<0.05$) in the levels of CRF_1-R in LC of LH rats as compared to the NC. Protein levels are expressed as ratio of intensity of the immunoreactive band for CRF_1-R normalized by the band for the loading control, GAPDH, and presented as mean±S.E.M. and * indicates significantly different from NC, RSS, and NH groups, $p<0.05$.

4.2.6. Repeated forced swim stress induced alterations in levels of GRK3, α_{2A}-AR and CRF_1-R in LC of rats can predict LH behavior in rats.

Data from biochemical analysis of GRK3, α_{2A}-AR, or CRF_1-R levels in LC of repeated force swim stress induced rats collectively were subjected to k-means cluster analysis, independent of behavior, to identify the presence of two clusters. The clusters were subjected to one-way ANOVA for statistical comparison with each other and with the non-swim controls. Statistically different clusters were identified. Then the data in these biochemical clusters were subjected to matching with the LH and NH rats that were determined from k-means cluster analysis of the behavioral data in the escape task. The percentage matching of clusters from biochemistry and escape testing analysis were used to compare the accuracy of prediction of escape behavior on the basis of the biochemistry in rats exposed to repeated forced swim stress.

The k-means cluster analysis of biochemical data collectively for GRK3, α_{2A}-AR, and CRF_1-R levels, in LC of rats exposed to repeated forced swim stress indentified two statistically different clusters. On matching with the behavior data, it was found that all the animals that were clustered as LH showed a decrease in GRK3 and an increase in α_{2A}-AR, and CRF_1-R. However, one animal that was behaviorally clustered as NH showed a decrease in GRK3 and increases in α_{2A}-AR and CRF_1-R. Thus there was 90% cluster symmetry between the clusters

identified on the basis of biochemistry and those identified on the basis of behavior.

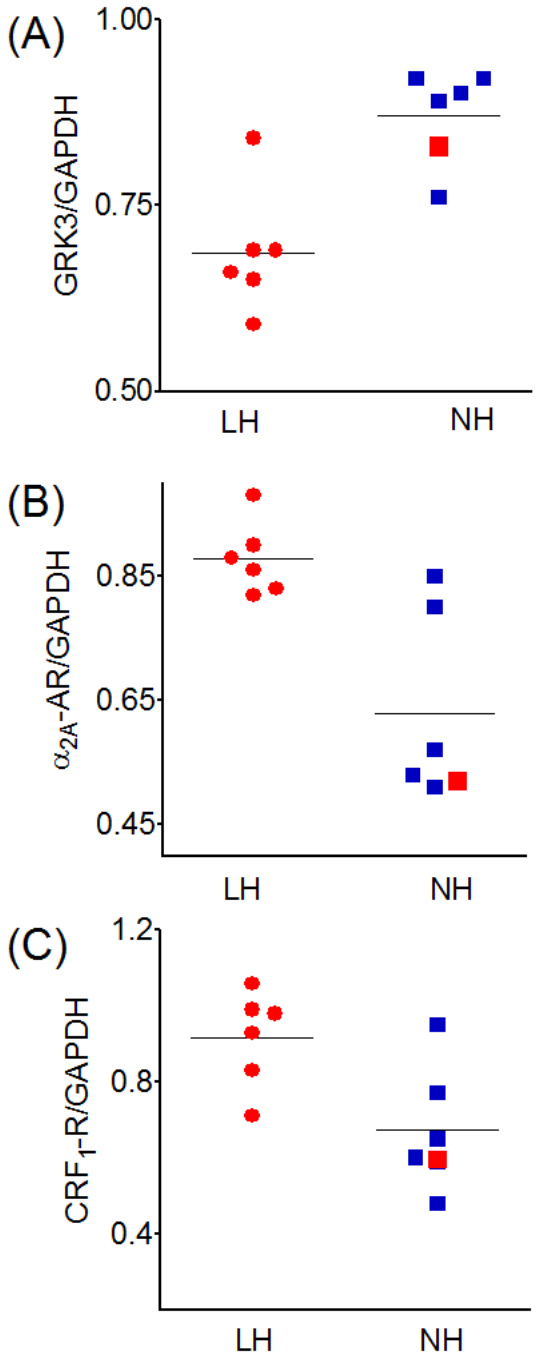
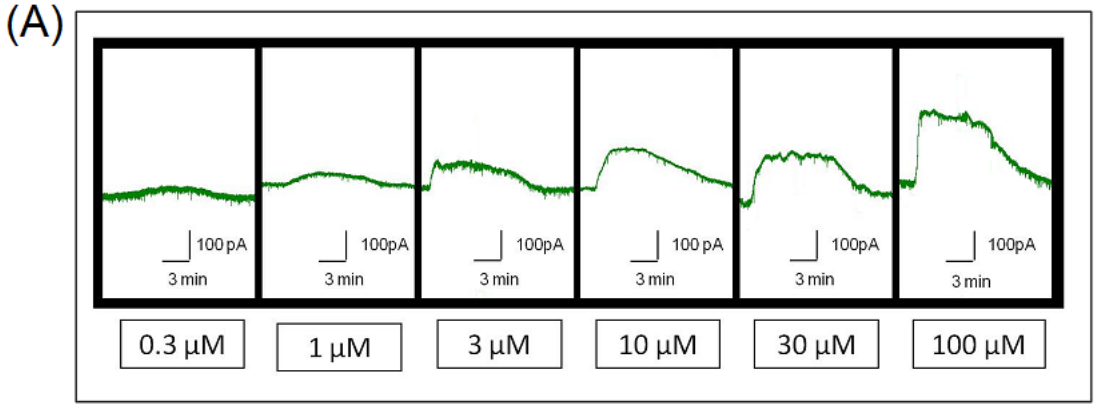

(A)
1.00
0.75
0.50
GRK3/GAPDH
LH
NH
(B)
0.85
0.65
0.45
α_{2A}-AR/GAPDH
LH
NH
(C)
1.2
0.8
0.4
CRF_1-R/GAPDH
LH
NH

Figure 21: There was high cluster symmetry between clusters identified by biochemical analysis and by behavior of rats exposed to RFS.
Rats were exposed to repeated forced swim stress and then the data obtained from biochemical analysis of GRK3, α_{2A}-AR, and CRF_1-R levels, collectively, in LC of rats exposed to repeated forced swim stress were subjected to k-means cluster analysis. Similarly the data from escape testing was subjected to k-means cluster analysis. The clusters obtained from biochemistry were matched with those obtained from the behavioral data to see the symmetry between them.
Panel (A, B, C) Symmetry matching of clusters identified from escape behavior with clusters identified from collective GRK3, α_{2A}-AR, and CRF_1-R data, respectively. Circles in each panel represent LH data points. Squares represent the NH data points. The colors blue and red represent the clusters identified by k-means cluster analysis of the collective biochemical data. There was 90% symmetry between the clusters identified by biochemistry and escape behavior.

4.3. Electrophysiological effects of norepinephrine (NE) on LC neurons from rats exposed to repeated forced swim stress.

4.3.1. Response to NE is increased in LC neurons of rats exposed to repeated forced swim stress.

To assess the responsiveness of α_{2A}-ARs on LC neurons, the electrophysiological responses to NE were determined. NE caused a concentration-dependent (0.3 µM to 100 µM) increase in outward membrane current in LC neurons (Fig 22A). Repeated forced swim stressed (RFS) rats exhibited a significant increase in membrane current in response to lower concentrations (0.3 µM, 1 µM and 3 µM) of NE in the LC neurons compared to LC neurons from the normal control (NC) or the repeated sham swim control rats (RSS). The EC_{50} (effective concentration producing 50% of the maximal response) for repeated forced swim stress rats, 1.8 µM, was significantly lower ($p<0.05$) than the EC_{50} for the non-swim control (25.0 µM) or the repeated sham swim control (7.2 µM) (Fig 22B). The maximum currents produced by NE across the groups were not significantly different.


(A)
100 pA
3 min
100pA
3 min
100pA
3 min
100 pA
3 min
100pA
3 min
100pA
3 min
0.3 µM
1 µM
3 µM
10 µM
30 µM
100 µM

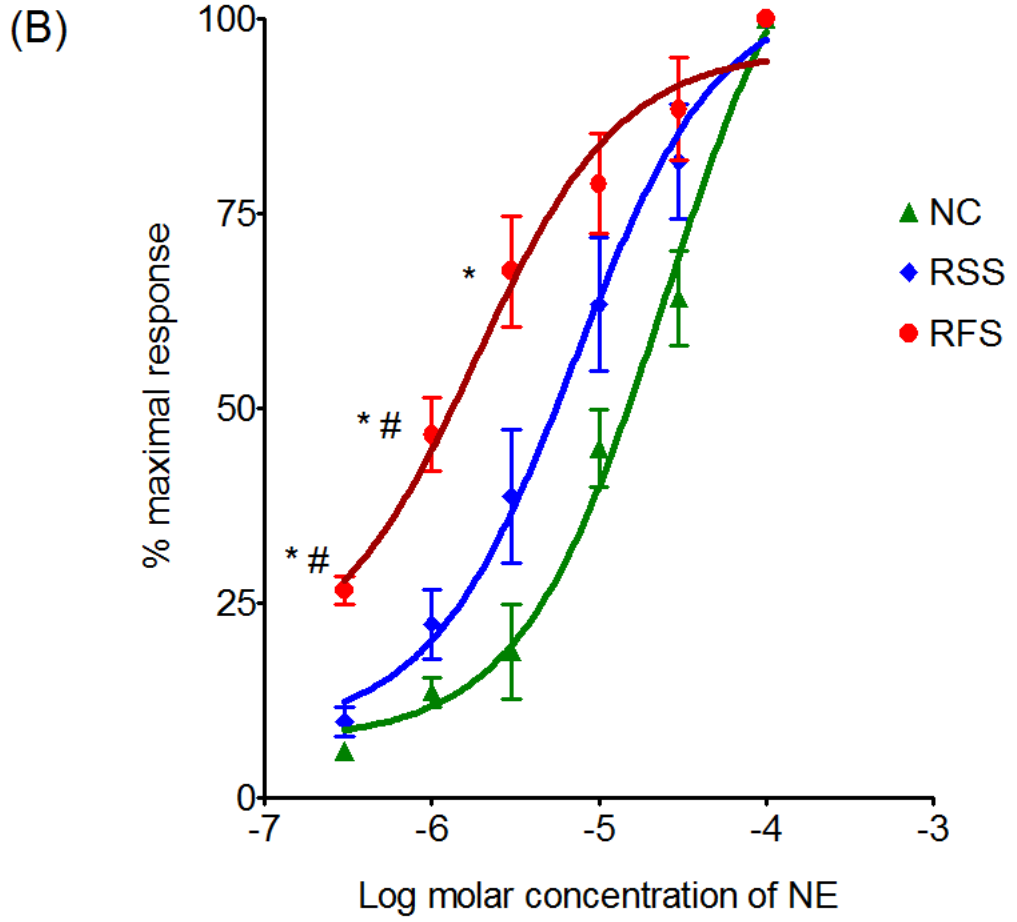

(B)
100
75
50
25
0
% maximal response
-7
-6
-5
-4
-3
Log molar concentration of NE
NC
RSS
RFS
*
* #
* #

Figure 22: Increased response to NE in the LC neurons of rats exposed to RFS.

Twenty-four hours after the last swim brains were removed and brainstem slices containing LC were prepared. Changes in membrane current in response to increasing concentrations of NE were recorded in voltage-clamp mode and used for analysis. The neurons were held at -60 mV holding potential. All the recordings were performed in the presence of cocaine (1 µM) and prazosin (100 nM) to inhibit neuronal NE uptake and inhibit alpha$_1$-adrenoceptor (α_1-AR) activation, respectively.

Upper panel – Representative traces displaying the increases in membrane current (pA) at increasing concentrations (µM) of NE in LC neurons from normal control rats.

Lower panel - Concentration-response curves for membrane current vs. NE concentration in LC neurons from the normal controls (NC, n=6), repeated sham swim (RSS, n=6), and repeated forced swim stress (RFS, n=6) rats. The results are presented as the response from each cell expressed as a percentage of the maximal response in that cell (mean ± SEM). * indicates significantly different from NC while # indicates significantly different from RSS, $p < 0.05$.

4.3.2. Identification of LC neurons from which the recordings were performed.

At the conclusion of each recording, neurobiotin was injected into each neuron using the recording electrode. The slices were then prepared for neurobiotin staining. The morphological characteristics of each neuron recorded from and their anatomical location within the slice helped confirm the LC location of each neuron. Representative photomicrographs of two of these LC neurons are provided (Fig 23).

4.3.3. Measurement of neuronal input resistance

The input resistance of each neuron was measured both prior to the NE concentration response measurements and at the end of the experiment. First, the current (I) **/** voltage (V) plots were generated using current steps. Then linear regression was used to fit the data to a straight-line equation. The slope of the line gave the input resistance. Representative I/V traces using step currents (Fig 24A) and the resulting I/V plots are provided in Fig 23B. Only those neurons where the input resistance did not change more than 15% during the course of the experiment were selected for analysis. Using this criterion about 20% of the data was excluded.

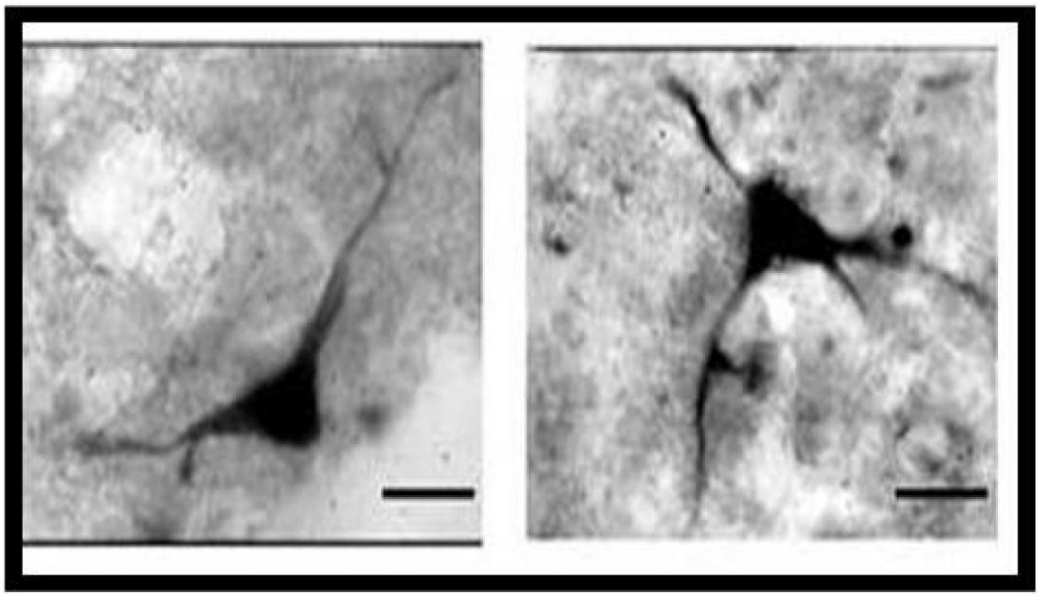
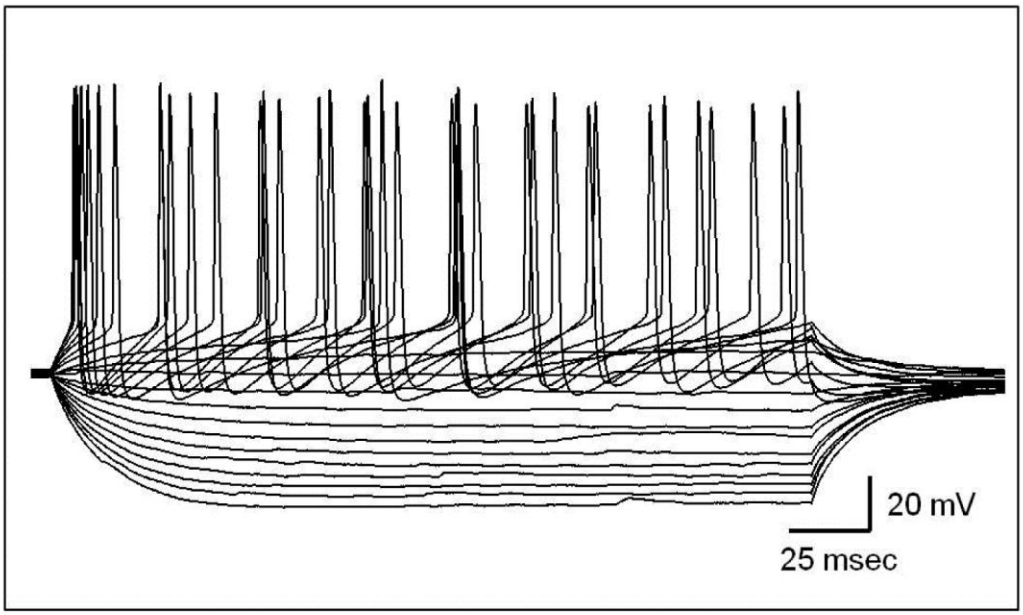


Figure 23: Confirmation that electrophysiological recordings were performed in neurons located within the LC.
The figure displays neurons from LC that had been stained with neurobiotin after whole-cell recording in a 300μM thick coronal section from the brain stem of rats. The whole-cell recordings were performed using microelectrodes filled with neurobiotin (2.5mg/ml). The slices containing the neurobiotin filled cells were later fixed and processed in avidin-biotin horseradish peroxidase solution as described in the methods. The slices were then developed to identify the neurobiotin staining. This helped confirm that the recorded neurons were within LC, by noting their anatomical location in the slice and their morphology. The scale bar represents 25 μm.

(A)



(B)

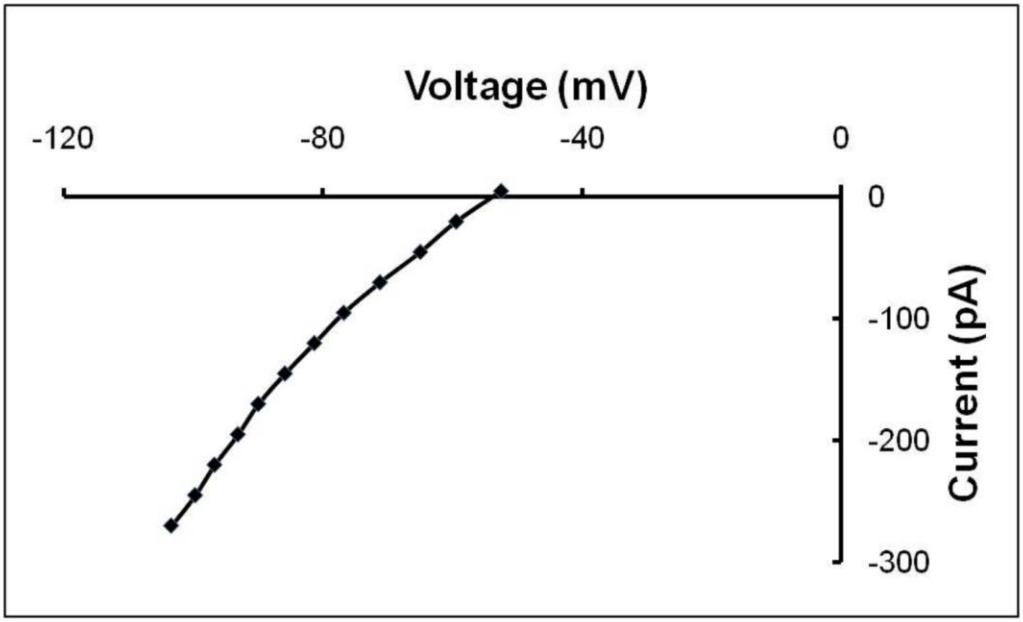


Figure 24: Representative current-voltage relationship in a LC neuron.
Input resistance of each neuron was estimated before the start and at the end of the experiment. This was done in the current-clamp mode by passing depolarizing step currents starting at -250pA for 250msec with an increment of 25pA. The resulting steady-state voltage was measured to obtain a current (I) – voltage (V) relationship by plotting an I/V values. Linear regression was used to fit to this I/V plot to a straight-line equation and input resistance was calculated from the slope of the line.
Panel A - Resulting membrane potentials to square wave current steps.
Panel B - The corresponding I/V plot.

4.4. Repeated forced swim stress did not cause any change in behavior and proteins in LC of the rats pretreated with DMI

4.4.1. Repeated forced swim stress did not cause any increase in immobility in rats pretreated with DMI.

Rats were treated with desipramine (5 mg/kg, bid) for a total of 10 days and subjected to repeated forced swim stress on the 8^{th}, 9^{th} and 10^{th} days. The immobility counts during the forced swim in the desipramine-treated rats were significantly lower than the non-treated rats on all the three days ($p<0.05$) (Fig 25A). There was no increase in immobility on day 3 compared to day 1 or day 2 in the rats treated with desipramine compared to that observed in the non-treated rats (Fig 25A). Finally, on all 3 days of forced swim stress, there was a significant increase in climbing behavior with desipramine treatment compared to the non-treated rats (Fig 25B). These results suggest that desipramine pretreatment prevented the despair behavior during repeated forced swim stress. The decrease in swimming behavior that was observed on day 3 in the non-treated rats also was abolished with desipramine treatment (Fig 25C).

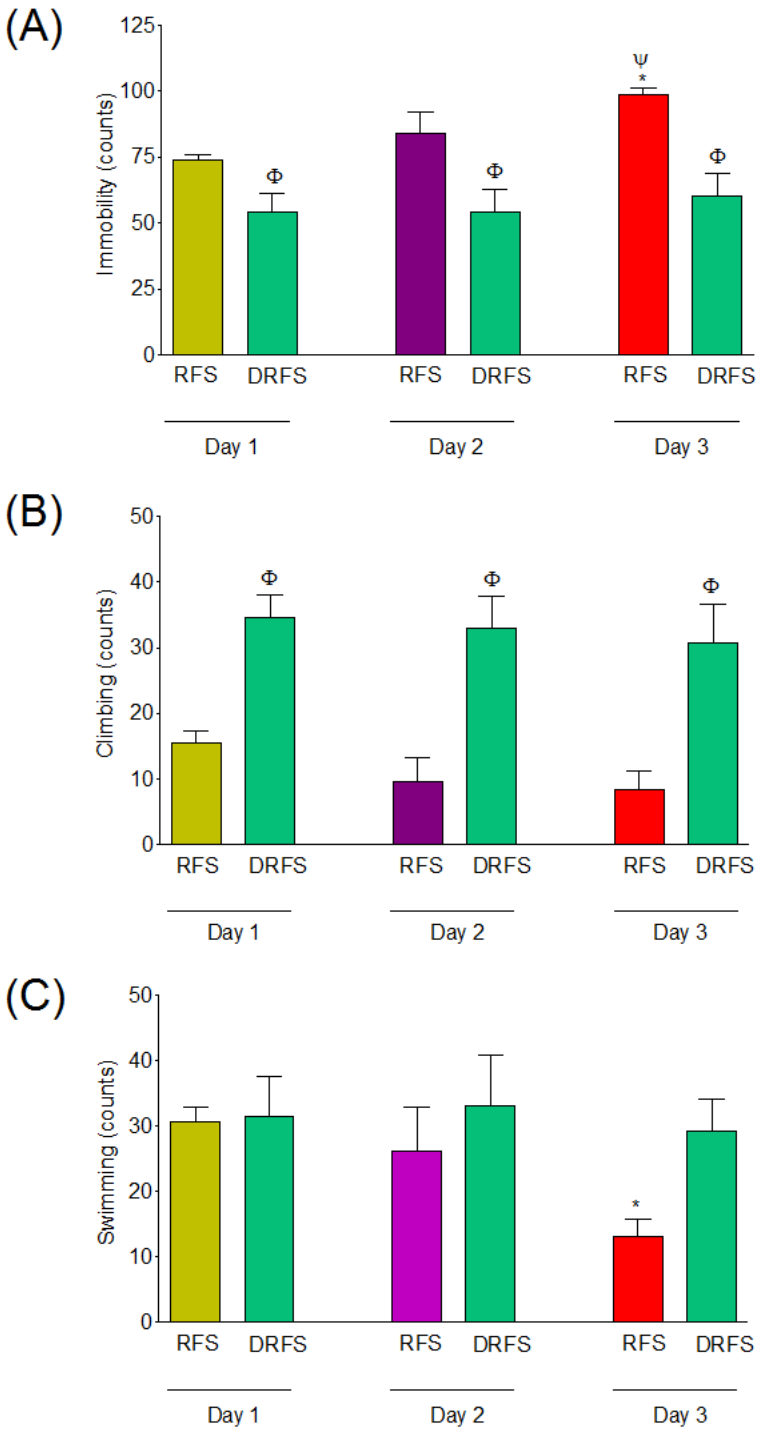

(A)
Immobility (counts)
125
100
75
50
25
0
Φ
Φ
Ψ
*
Φ
RFS
DRFS
RFS
DRFS
RFS
DRFS
Day 1
Day 2
Day 3
(B)
Climbing (counts)
50
40
30
20
10
0
Φ
Φ
Φ
RFS
DRFS
RFS
DRFS
RFS
DRFS
Day 1
Day 2
Day 3
(C)
Swimming (counts)
50
40
30
20
10
0
*
RFS
DRFS
RFS
DRFS
RFS
DRFS
Day 1
Day 2
Day 3

Figure 25: Repeated forced swim stress did not cause any increase in immobility in the rats pretreated with DMI.

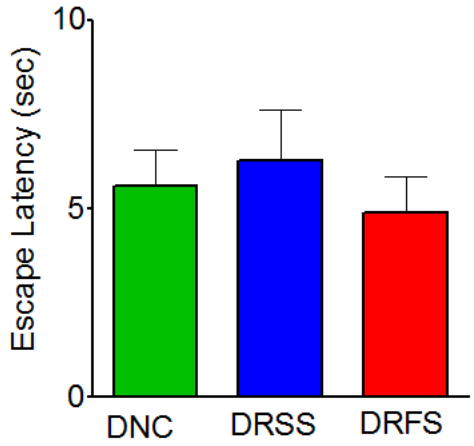
Rats were treated with desipramine (DMI, 5 mg/kg, bid) for a total of 10 days and subjected to repeated forced swim stress on the 8^{th}, 9^{th} and 10^{th} days. The behavioral parameters analyzed were swimming (C), climbing (B) and immobility (A). The predominant behavior (swimming, climbing or immobility) observed during each 5 sec period was scored by blinded observer. The total number of counts for each behavior that was scored, in the first 10 min of swim, was noted. Mean counts for each group for each of three days were determined. DMI-treated repeated forced swim stress rats (DRFS) showed reduced immobility and increased climbing compared to untreated repeated forced swim stressed rats (RFS), on all the three days. The data are presented as mean±S.E.M. of the number of periods for immobile behavior. * and Ψ indicate significantly different from day 1 and day 2, respectively, $p<0.05$. Φ indicates significantly different from the non-treated forced swim stressed group, $p<0.05$

4.4.2. Repeated forced swim stress did not induce changes in escape behavior in shuttle-box in the rats pretreated with DMI.

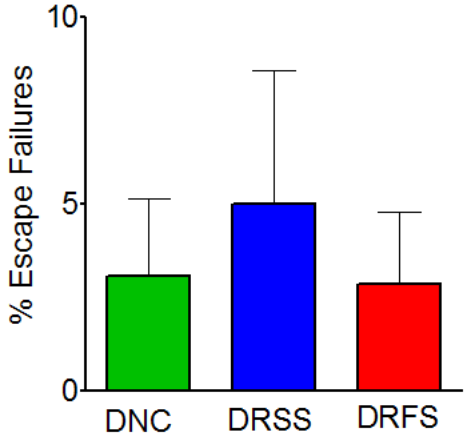
Rats were treated with desipramine (5 mg/kg, bid) for a total of 10 days and subjected to repeated forced swim stress on the 8^{th}, 9^{th} and 10^{th} days. Then, 24 hr after the last swim, they were tested for escape behavior in the shuttle-box. In the 5 fixed ratio 1 (FR-1) trials, which were used to rule out any motor deficit in the rats, there were no differences in the mean escape latency / percentage escape failure between any of the desipramine-treated non-swim control (DNC) , repeated sham swim control (DRSS) or repeated forced swim stressed (DRFS) groups (Fig 26 A, B). Also, there were no differences in the mean escape latency / percentage escape failure between any of the groups in the fixed ratio 2 (FR2) trials (Fig 26 C, D). Additionally, when K-means cluster analysis was applied to the behavior data (mean escape latency and percentage escape failure) from the DFS group, the data did not showed any bifurcation into two significantly different clusters based on the mean escape latency or percentage escape failure (Fig 26 C, D). This indicates that the desipramine treatment abolished the repeated three days forced swim stress-induced increase in escape latency and increased escape failures observed in the fixed-ratio 2 trials of the untreated rats.

FR1

(A)

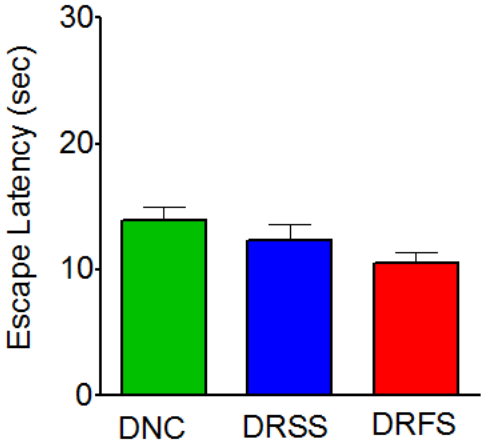

Escape Latency (sec)
10
5
0
DNC
DRSS
DRFS

(B)


% Escape Failures
10
5
0
DNC
DRSS
DRFS

FR2

(C)


Escape Latency (sec)
30
20
10
0
DNC
DRSS
DRFS

(D)

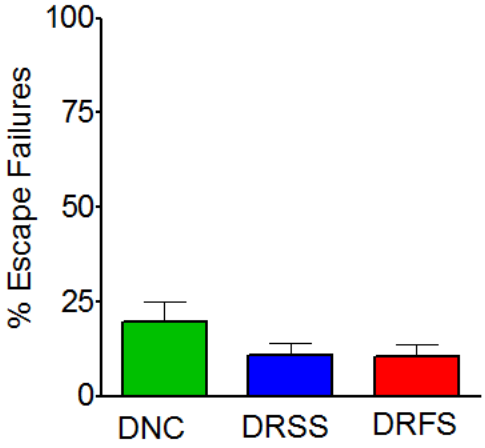
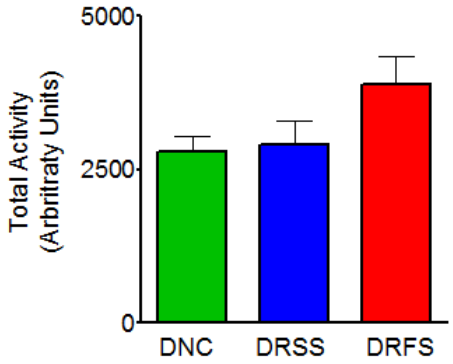

% Escape Failures
100
75
50
25
0
DNC
DRSS
DRFS

Figure 26: Repeated forced swim stress did not induce any changes in escape behavior of rats pretreated with DMI in the shuttle-box.
Rats were treated with desipramine for a total of 10 days and forced swim stressed on the 8^{th}, 9^{th} and 10^{th} days. Twenty-four hours after the last swim all the rats from desipramine treated non-swim control (DNC, n=12), repeated sham swim control (DRSS, n=12) and repeated forced swim stress (DRFS, n=12) groups were tested for escape behavior in the shuttle-box. The parameters tested were escape latency and percentage escape failure. When k-means cluster analysis was applied to the DRFS data, there was no bifurcation into two significantly different clusters based on the mean escape latency or percentage escape. Additionally, escape latencies and % escape failures from DRFS were not different from the controls, DNC and DRSS.
Panel (A) –Mean escape latencies for DNC, DRSS and DRFS rats for the fixed ratio-1 trials.
Panel (B) – Percentage escape failures for DNC, DRSS and DRFS rats for the fixed ratio 1 trials.
Panel (C) – Mean escape latencies for DNC, DRSS and DRFS rats for the fixed ratio-2 trials.
Panel (D) – Percentage escape failures for DNC, DRSS and DRFS rats for the fixed ratio-2 trials. Data are presented as mean±S.E.M.

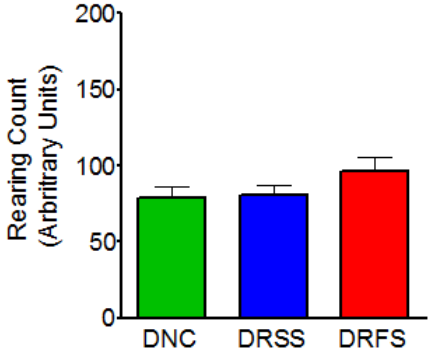
4.4.3. Open field test

There were no significant differences in the locomotor activity, assessed by total activity, between any of the groups (Fig 27a). Also, exploratory behavior of the rats, assessed by their rearing counts (Fig 27B), did not demonstrate any significant differences between DNC, DRSS and DRFS rats. Anxiety behavior also was evaluated from the open field test. This was done by calculating the percentage of total time spent by rats in the central compartment. There were no significant differences amongst DNC, DRSS and DRFS rats for this parameter (Fig 27C).

(A)


Total Activity
(Arbritraty Units)
5000
2500
0
DNC
DRSS
DRFS

(B)


Rearing Count
(Arbritrary Units)
200
150
100
50
0
DNC
DRSS
DRFS

(C)

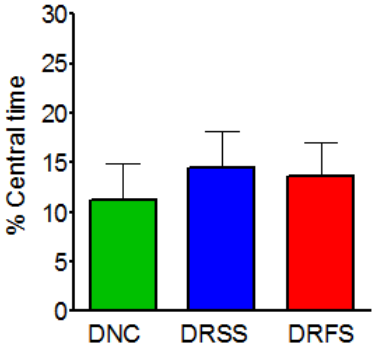

% Central time
30
25
20
15
10
5
0
DNC
DRSS
DRFS

Figure 27: DMI pretreatment did not cause any difference in the level of locomotor and exploratory activity or anxiety behavior in the Open Field Test between the DNC, DRSS and DRFS rats.
One day after the escape testing the DNC (n=12), DRSS (n=12) and DRFS (n=12) rats were subjected to the open field test to assess locomotor and exploratory activities as well as to assess anxiety.
Panel A - Exploratory activity as indicated by total activity.
Panel B - Locomotor activity as indicated by rearing counts.
Panel C - Anxiety behavior as indicated by % central time. Data are presented as mean±S.E.M. No significant differences were found.

4.4.4. Elevated plus Maze test

The DNC, DRSS and DRFS rats were subjected to the elevated plus maze test to assess anxiety-related behavior. No differences between the DNC, DRSS and DRFS rats were observed in the anxiety parameters (Fig 28). This test was used as a control to observe if the effects of repeated forced swim stress on escape task had any confounding anxiety component.

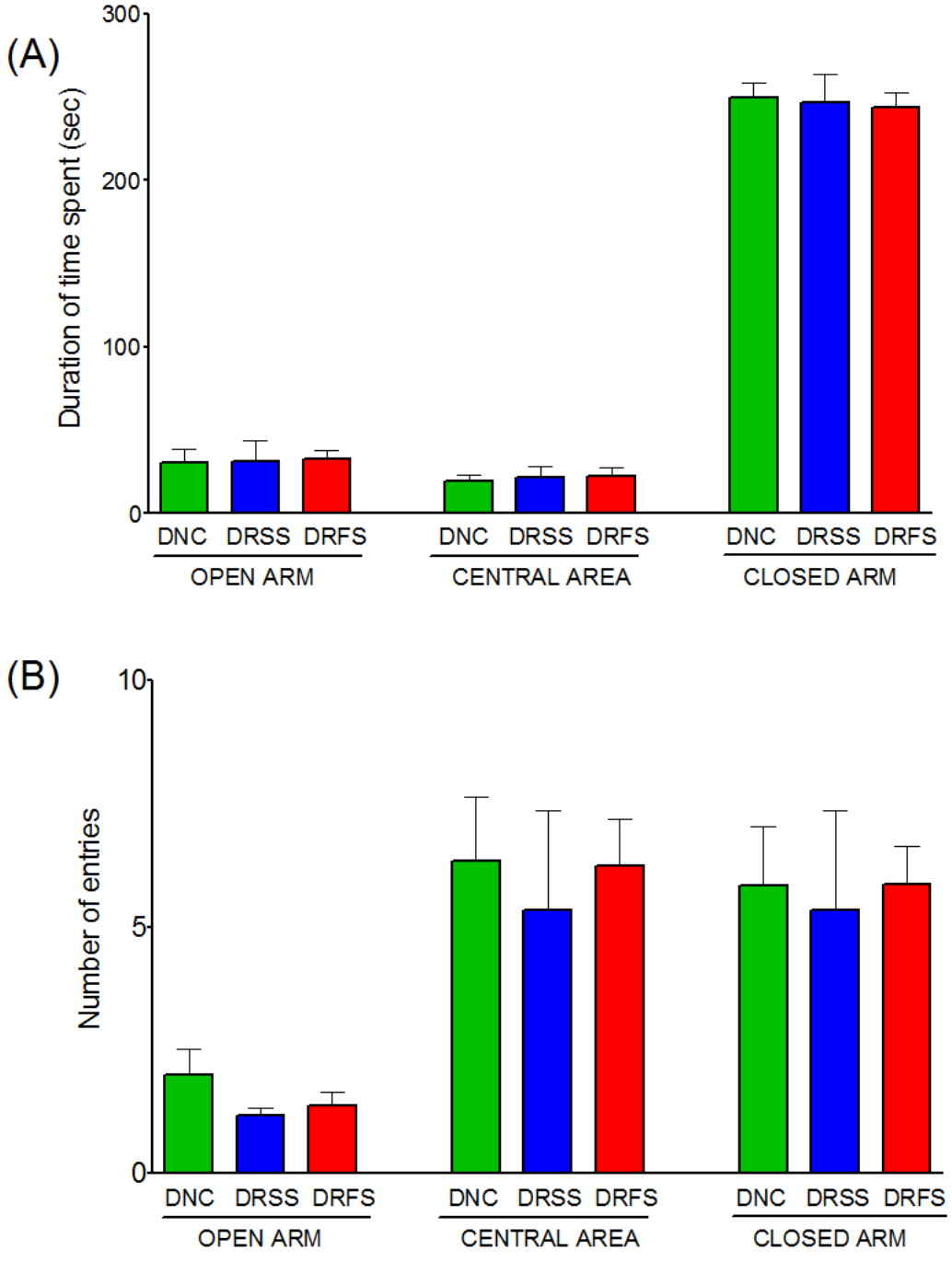

(A)
Duration of time spent (sec)
300
200
100
0
DNC DRSS DRFS
OPEN ARM
DNC DRSS DRFS
CENTRAL AREA
DNC DRSS DRFS
CLOSED ARM
(B)
Number of entries
10
5
0
DNC DRSS DRFS
OPEN ARM
DNC DRSS DRFS
CENTRAL AREA
DNC DRSS DRFS
CLOSED ARM

Figure 278: DMI pretreatment did not cause any differences in performance of the DNC, DRSS and DRFS rats in the Elevated Plus Maze test for anxiety behavior.

One day after the escape testing the DNC (n=12), DRSS (n=12) and DRFS (n=12) rats were subjected to the elevated plus maze to assess anxiety.

Panel A – Duration of time spent by the animals in open arm, central area and the closed arm.

Panel B - Number of entries undertaken by the animals into the open arm, central area and the closed arm. The performance of all rats was similar in all the parameters investigated. Data are presented as mean±S.E.M. No significant differences were found.

4.4.5. Repeated forced swim stress did not cause any change in levels of GRK3, GRK2, TH, α_{2A}-AR and CRF_1-R in LC of rats pretreated with DMI.

There were no changes in the levels of GRK3 (Fig 29), GRK2 (Fig 30), TH (Fig 31), α_{2A}-AR (Fig 32) and CRF_1-R (Fig 33) in LC of DRFS rats compared to DNC and DRSS rats.

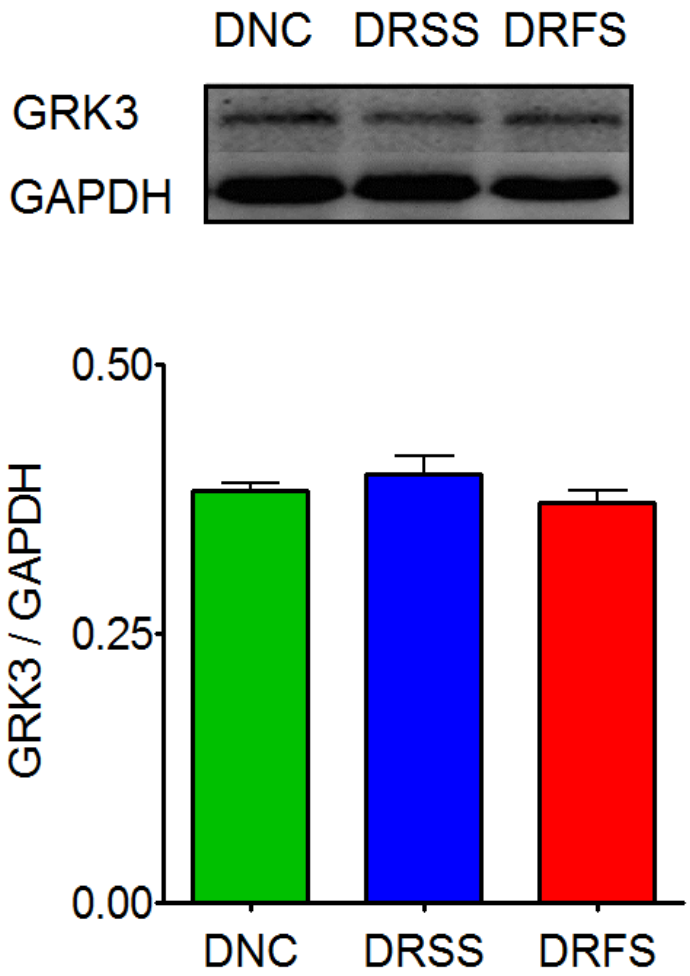


Figure 289: Repeated forced swim stress did not cause any change in levels of GRK3 in LC of rats pretreated with DMI.
Rats were treated with DMI for a total of 10 days and forced swim stressed on the 8th, 9th and 10th days. Twenty-four hours after the escape testing the brains from DNC (n=12), DRSS (n=12) and DRFS (n=12) rats were harvested and levels of GRK3 were determined by western blot analysis. The figure displays representative western blots and mean levels of GRK3 in DNC, DRSS and DRFS groups. Data are presented as mean±S.E.M. No differences were observed between the groups.

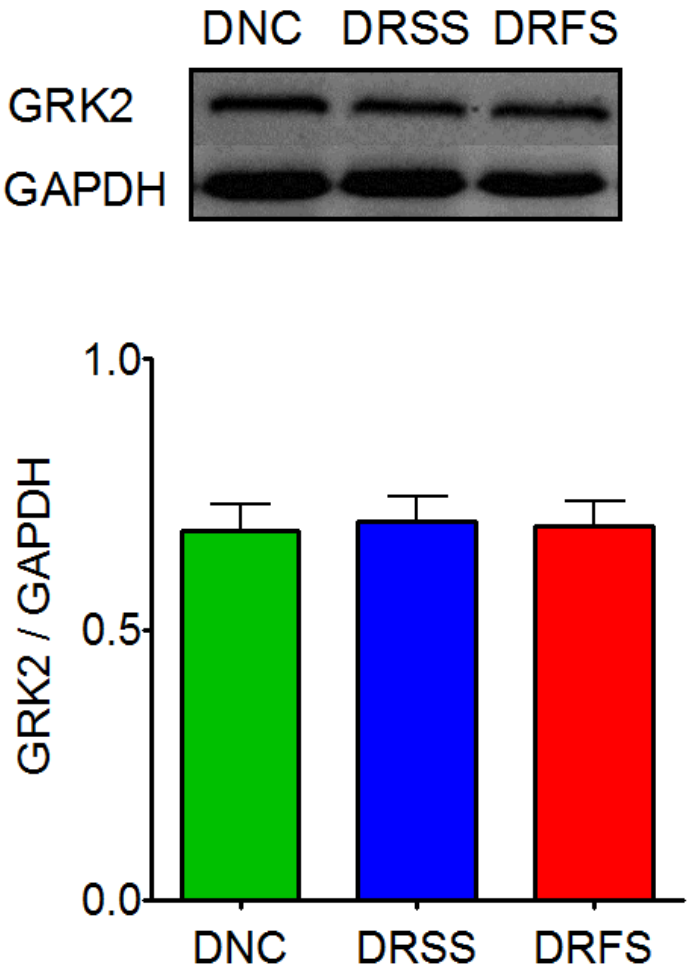


Figure 30: Repeated forced swim stress did not cause any change in levels of GRK2 in LC of rats pretreated with DMI.
Rats were treated with DMI for a total of 10 days and forced swim stressed on the 8th, 9th and 10th days. Twenty-four hours after the escape testing the brains from DNC (n=12), DRSS (n=12) and DRFS (n=12) rats were harvested and levels of GRK2 were determined by western blot analysis. The figure displays representative western blots and mean levels of GRK2 in DNC, DRSS and DRFS groups. Data are presented as mean±S.E.M. No differences were observed between the groups.

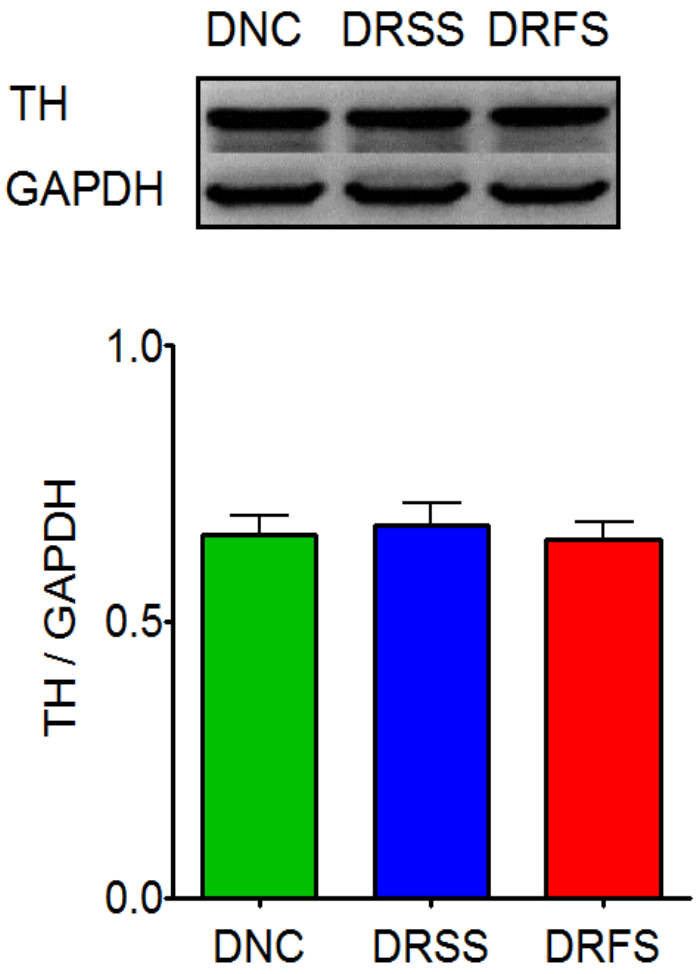


Figure 31: Repeated forced swim stress did not cause any change in levels of TH in LC of rats pretreated with DMI.
Rats were treated with DMI for a total of 10 days and forced swim stressed on the 8th, 9th and 10th days. Twenty-four hours after the escape testing the brains from DNC (n=12), DRSS (n=12) and DRFS (n=12) rats were harvested and levels of TH were determined by western blot analysis. The figure displays representative western blots and mean levels of TH in DNC, DRSS and DRFS groups. Data are presented as mean±S.E.M. No differences were observed between the groups.

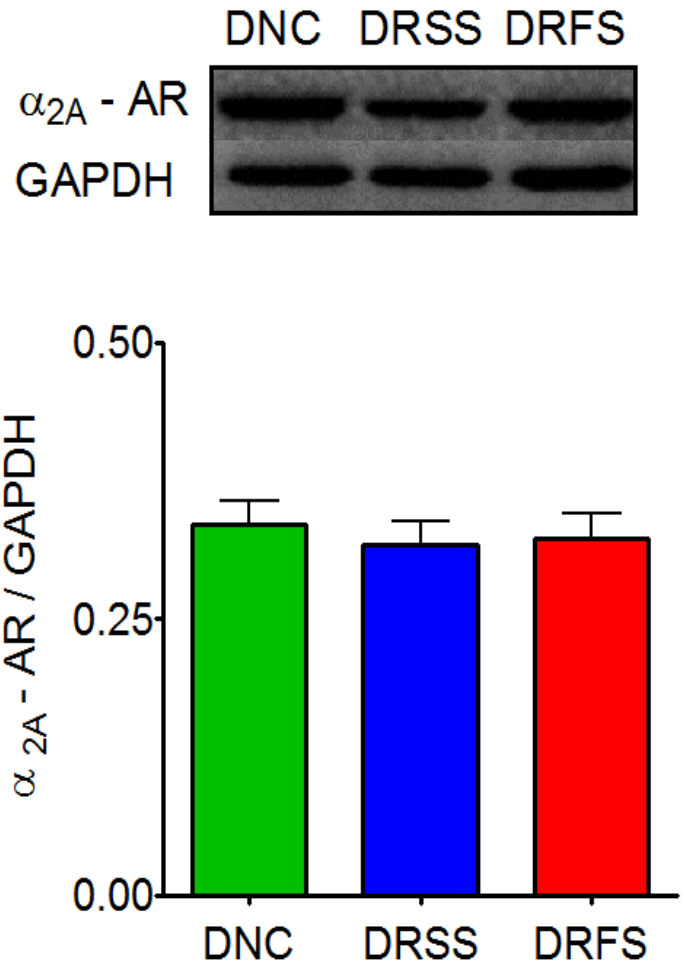


Figure 32: Repeated forced swim stress did not cause any change in levels of α_{2A}-AR in LC of rats pretreated with DMI.
Rats were treated with DMI for a total of 10 days and forced swim stressed on the 8th, 9th and 10th days. Twenty-four hours after the escape testing the brains from DNC (n=12), DRSS (n=12) and DRFS (n=12) rats were harvested and levels of α_{2A}-AR were determined by western blot analysis. The figure displays representative western blots and mean levels of α_{2A}-AR in DNC, DRSS and DRFS groups. Data are presented as mean±S.E.M. No differences were observed between the groups.

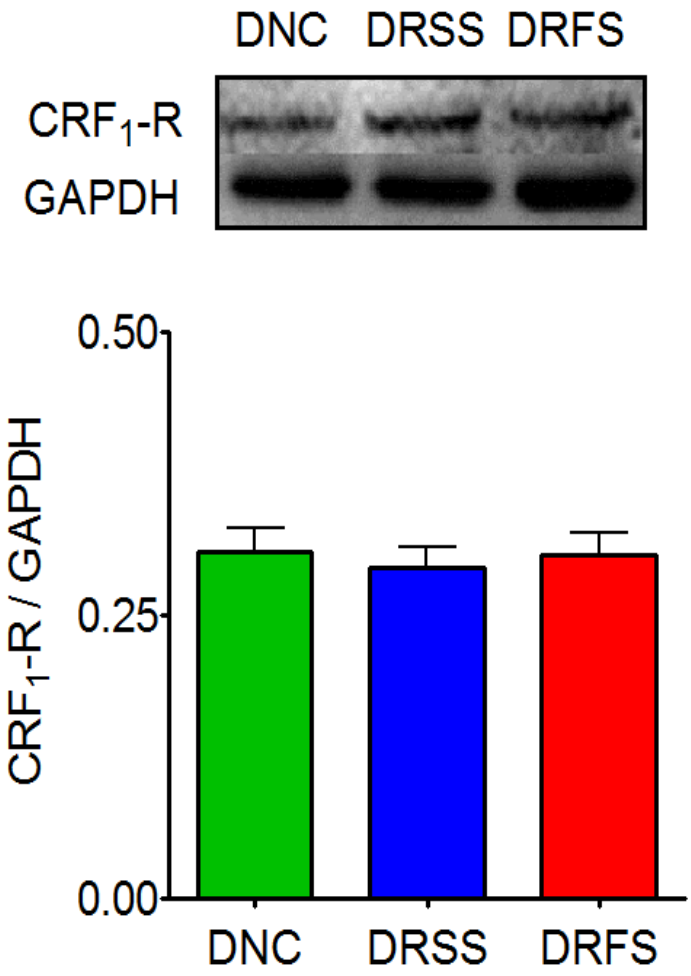


Figure 33: Repeated forced swim stress did not cause any change in levels of CRF_1-R in LC of rats pretreated with DMI.
Rats were treated with DMI for a total of 10 days and forced swim stressed on the 8th, 9th and 10th days. Twenty-four hours after the escape testing the brains from DNC (n=12), DRSS (n=12) and DRFS (n=12) rats were harvested and levels of CRF_1-R were determined by western blot analysis. The figure displays representative western blots and mean levels of CRF_1-R in DNC, DRSS and DRFS groups. Data are presented as mean±S.E.M. No differences were observed between the groups.

4.5. Repeated forced swim stress – 7 days.

4.5.1. Increasing the repeated forced swim stress from 3 to 7 days did not further increase the immobility behavior.

Rats were subjected to repeated forced swim stress for seven days. The behavioral parameter analyzed during the swim stress was immobility (passive behavior which reveals a despair condition). Repeated forced swim stress caused an increase in immobility counts during the first 10 min of day 3 compared to day1 or day 2 ($p<0.05$) (Fig 34). However, starting from 4^{th} day onwards the rats started to support themselves with their tail and paws along the walls of the plexiglass cylinder and on the surface of the water. Although the rats were immobile and were not showing any active behavior this posture was distinctly different from the immobility behavior that is considered indicative of despair that was observed on days 1-3.

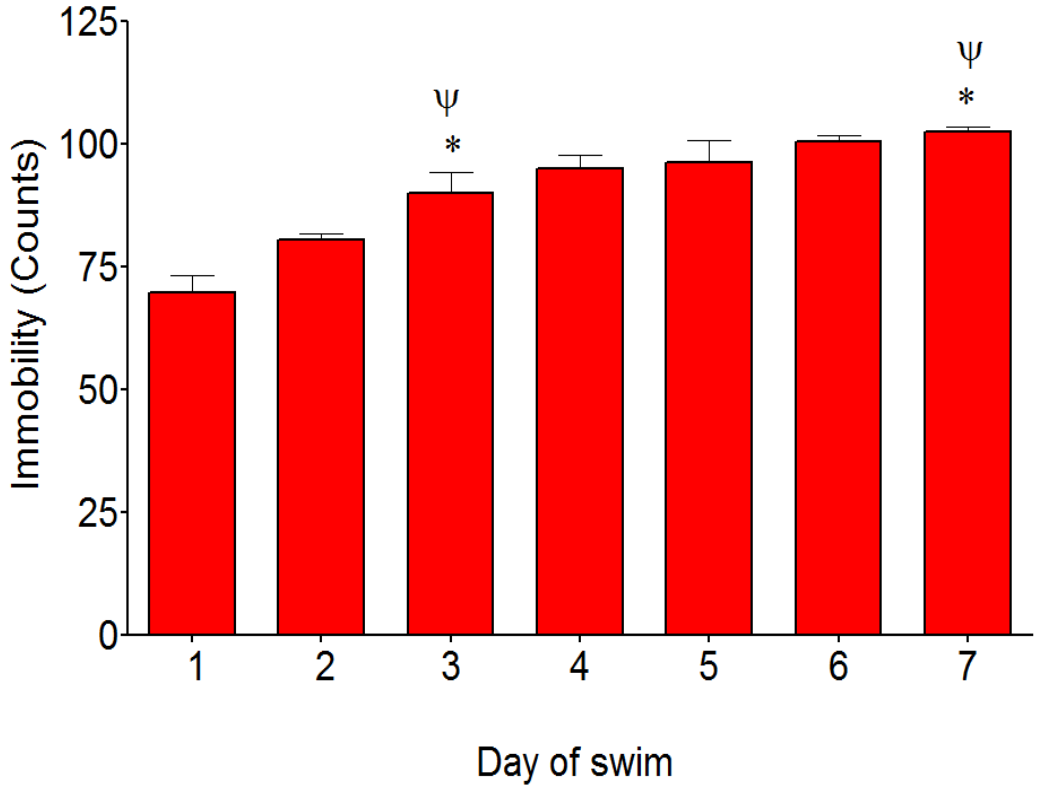


Figure 294: Repeated forced swim stress for 7 days did not cause any further increase in immobility behavior than that caused by repeated forced swim stress for 3 days

Rats (n=8) were subjected to repeated forced swim stress for seven days by exposing them to 30 cm deep water for 10 min on day 1 and 20 min each on day 2 to day 7. The behavioral parameters analyzed were immobility. The predominant behavior (swimming, climbing or immobility) observed during each 5 sec period was scored by a blinded observer. The total number of counts for each behavioral that was scored, in the first 10 min of swim, was noted. Mean count for each group for each of three days was determined.

Panel - Immobility counts on days 1to 7 of forced swim stress. The data are presented as mean±S.E.M. of the number of immobility counts for each day and * and Ψ indicates significantly different from day 1 and day 2, respectively, $p<0.05$.

5. DISCUSSION

5.1. Single and repeated forced swim stress induces Learned Helplessness.

The inability of an animal to control being exposed to stress plays an important role in the exhibition of the impaired escape behavior called LH (178). Various researchers have reasoned that the animal's inability to control the hostile condition is a key component in the subsequent appearance of an impaired escape behavior in behavior tests (61, 179). An important attribute of a stressor that cannot be controlled is that it modifies the manner in which the animal reacts to challenges that are different from the original stress (61, 180). Until now, predominantly electric shock (foot shock or tail shock) has been used as a stressor to induce LH behavior. One objective of the present study was to determine if another stress paradigm different from the electric shock, namely forced swim stress, could induce LH behavior in animals, as swim stress is an inescapable and uncontrolled stressor (181, 182).

A previous report noted that there was a moderate impairment of escape behavior in the shuttle-box after exposure to an inescapable swim stress (174). However, their paradigm was different from the one used in the present study or the one typically used for forced swim stress. The mentioned report used an

intermittent type of swim stress where the rats were subjected to one hundred 5s swims over roughly 100 minutes for the complete paradigm and in a single day. This was equal to about 7 min total swim time as compared to the single swim stress paradigm used in the present study wherein the animal was in the water continuously for 10 minutes. In the previous study the investigators noted that although they observed escape deficits in shuttle-box testing, the deficits were not as robust as deficits resulting from tail shock stress. They hypothesized that modifying the swim stress parameters might amplify the escape deficits (174), but this was never tested. The results presented herein clearly demonstrate that repeated forced swim stress can impair escape behavior to an extent comparable to that observed with electric shock.

An additional important attribute of using forced swim as a stressor is that the context in which the animals are tested for escape behavior will be totally different from the context of the original stress. This has an important implication. Classically two different variations of the learn-helplessness paradigm have been used. In one, animals are subjected to inescapable and uncontrollable electric shock (stressor) and tested for escape behavior in the same context (meaning that both stress paradigm and escaping testing were done in the shuttle-box) (158, 183-185). In another method the animals are stressed in a different context (using tail shock in a restrainer) from the escape behavior testing (done in the shuttle-box) (158, 186). Reportedly, when the stress and testing for escape

behavior are done in a different context (meaning that the animals had no related cues to the stress in the test environment for escape behavior), the duration of escape behavior deficits is short (3-4 days). In contrast to this, when the stress exposure and escape testing were done in the same context (in the shuttle-box), the behavioral impairment lasted weeks. It was argued that using a similar context for the stress and escape testing resulted in an interfering contextual fear (158, 186), as opposed to the paradigm where the stress and testing are done in distinct contexts (158, 186). In the present study, where the swim stress is in a plexiglass cylinder and escape behavior is tested in shuttle-box, the possibility of a confounding contextual fear is eliminated. The stressor itself also is completely different compared to the electric shock which was used for both the stressing and the escape task in the classical LH paradigm.

In the present study the rats were first exposed to a single forced swim stress and twenty-four hours later they were subjected to testing for escape behavior in a shuttle-box. The averages of the measurement of the escape latency and % escape failures in the single forced swim stress group, taken as a whole, did not show any differences from the controls. In contrast a more robust stressor, a single bout of 100 electric tail shocks over 100 min, results in statistically significant behavioral deficits for the total stressed group in the escape task data (unpublished results from our lab; (187)). Therefore, the milder stressor employed herein, a single bout of forced swim, does not appear, on first

examination to produce detectable escape deficits. However there was high degree of heterogeneity in the escape data. When the data from the single forced swim stressed population of rats is subjected to k-means cluster analysis, the data bifurcated into two significantly different groups. One group showed significant escape deficits (LH) compared to the controls and the other group showed escape behavior similar to the controls (NH). Thus, since some but not all rats showed escape deficits, only after cluster analysis could the susceptibility of some rats to this milder stress be revealed. Collectively, these results support the potential universality of inescapability in the induction of behavioral deficits in rats that have increased susceptibility to the adverse consequences of stress, be the stress mild or more severe.

When rats were subjected to repeated forced swim stress daily for three days, immobile behavior (passive behavior) during the swim increased progressively during from day to day. This is in agreement with a previously reported study using repeated forced swim stress (49). There are reports in the literature that NE release progressively increases in the terminal fields of LC neurons from day 1 to day 2 of exposure to swim stress, when the post stress NE in the terminal field was compared to the baseline prior to the stress on each day (188). A 183% increase in NE over the baseline was observed after a single swim and a 310% increase was observed after the second swim on next day (188). This suggests a progressively increased LC neuronal activity and

neurochemical changes with repeated exposure to swim stress. These changes in LC neuronal activity and neurochemistry have been argued to be associated with adverse symptoms of stress (21). In addition to the number of swim stress sessions and duration of the stressor, the environment in which the stressor is presented might also impact the overall consequences of the stress. For example, factors like the water temperature during the swim might contribute to the magnitude of the stress-initiated behavioral changes like increased immobility (189). To diminish the impact of cold stress in the present paradigm, the swim stress was performed in water maintained at 25ºC.

When the repeatedly stressed rats were subjected to escape testing 24 hr after the last swim, there was slightly but not significantly increased escape latency in the repeated forced swim stress group compared to controls. However, the three-day repeated forced swim stress group showed a significantly higher percentage of escape failure compared to controls. On subjecting the data from repeated forced swim stress group to k-means cluster analysis, the data revealed the existence of two significantly different clusters. One cluster showed significant greater escape latency and escape failures compared to controls and was termed LH while the other cluster showed escape behavior similar to the controls and was termed NH. An important issue that arises here is, how are the escape deficits induced by single forced swim stress different from those after repeated forced swim stress. Two questions that might highlight the differences

between the two stress protocols are: 1) does the incidence of animals becoming LH increase with repeated swim stress? or, 2) Does the magnitude of escape impairment increase with an increase in the “dose” of stress. The incidence of LH (50% of the rats that were subjected to forced swim stress) was similar after both single and repeated forced swim stress. However, the LH group after three days of repeated forced swim stress showed higher escape latency (27.1 second for three day stress versus 22.9 second for single stress) and higher escape failures (82.7% for three day stress versus 61% for single stress) than the rats subjected to a single day of forced swim stress. It might also be asked, if the dose of stress is increased (from single exposure to repeated exposure) then why didn’t the incidence of rats becoming LH increase. In the present study the conditions of the escape task was maintained the same for all rats (controls, single forced swim and repeated forced swim stressed). We suggest it is likely that the level of difficulty for the escape task was responsible for the equal distribution of the LH and NH rats. Moreover, it is possible that if the difficulty of the escape task was increased we would have observed a greater fraction of LH rats in the total stress rat (190).

To rule out any confounding effects on the induction of LH, other than the swim stress itself, the open field test and elevated plus maze tests were performed. The similar performances of the rats from all the groups in the open field test and the elevated plus maze test indicate that the stressed rats had

neither impaired locomotion nor increased anxiety. Therefore, these potentially interfering factors did not confound the LH behavior in the rats.

Rats also were subjected to seven days of repeated forced swim stress. However, while there was progressive increase in the immobility time during the first three days, the immobility time plateaued at and beyond day 3. Also, from the fourth day onwards the rats started to support themselves with paws and tail against the wall of the cylinder (Fig 35). This could not be considered a true immobile behavior in the sense that it was not a despair condition and rather can be viewed as a coping or adaptive mechanism rather than despair in response to an inescapable stressful situation. Thus the swim stress was no longer perceived as threatening by the rats. For this reason three days of repeated swim stress was selected as the stress paradigm after which we examined the effects of repeated swim stress on escape behavior and the associated neurochemical changes.

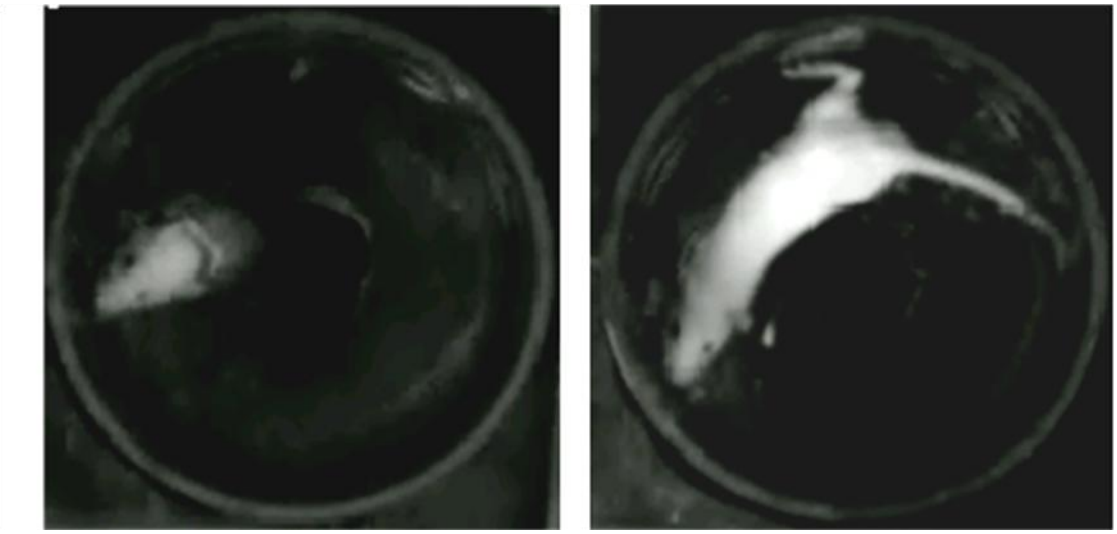


Figure 35: Immobility of rats on day 3 and day 7
The left panel shows immobile behavior of the rats on day 3 where the rats maintained minimum movement while giving up attempt to escape and this behavior was identified as “despair” or immobility. However, as shown in the right panel the rat learned to support itself against the walls using tail, paws and nose. Therefore, although it was not pursuing any active behavior, this behavior cannot be considered as “despair” or true immobility in its classical sense.

5.2. Repeated forced swim stress as a model of inducing behavioral impairment

One of the significant findings of the present study is that repeated forced swim stress, which is an inescapable and uncontrollable stress, induces escape deficits (termed LH). This escape deficit is a behavioral deficit that resembles symptoms of depressive behavior in humans and can be reversed by use of antidepressant drugs. Thus this model of repeated forced swim stress has good face validity, meaning it can mimic in animals the symptoms of the illness that are shown in humans. As mentioned above, this model excluded interference due to contextual fear as the stress paradigm and escape testing are done in completely different environments without any common phenomenon other than inescapability and uncontrollability.

The results of behavioral tests indicate a strong convergent and discriminant validity of this repeated forced swim paradigm as a model to induce escape deficits. Convergent validity of a model indicates the extent to which it is in agreement with results of other models that judge the same behavioral construct. The present paradigm of inescapable forced swim stress, like inescapable electric shock, induces behavioral deficits (called LH) in rats. Discriminant validity of a model indicates the extent to which a test measuring a particular feature of a phenomenon lacks correlation with measurements of

another test that judges a separate aspect of the phenomenon. In the present study the open-field test and elevated plus maze tests were performed to test for anxiety behavior that also is a stress-related behavior. No increase in anxiety behavior was observed after exposure to repeated forced swim stress, indicating that the model has good discrimant validity. The repeated forced swim stress also demonstrated a good predictive validity. Predictive validity is the degree to which a model can express an outcome of interest in advance, like for example, the effectiveness of a drug in treating a mental disorder. Desipramine prevented escape deficits induced by repeated forced swim stress highlighting the predictive validity of the model.

An additional advantage of this model of repeated forced swim stress is that it provides a more physiologically relevant stressful condition as opposed to the electric shock. While electric shock is considered more robust stress than swimming, it also is more artificial since it involves use of electrodes to shock the animals. Hence there is a need for a model which offers a milder and physiologically relevant stressful situation (swim stress) but wherein the stress is sufficient to enable identification of stress-susceptible and stress-resistant groups in a subsequent escape task. As such the repeated forced swim stress paradigm may have greater relevance as a model where the neurochemical and receptor regulation differences between stress-susceptible and stress-resistant populations can be characterized.

5.3. Repeated forced swim stress-induced changes in GRK3 in LC of Learned Helpless rats.

One questions that arises from the results of the behavioral studies is, what is different in the rats that show escape deficits, compared to rats that perform similar to the controls in the escape task after exposure to swim stress (single or repeated). Another is, what role if any does GRK3 in LC have in this regard?

First, let us consider what happens after exposure to single forced swim stress. Single forced swim stress caused no changes in the levels of GRK3 in the LC of rats compared to control rats when measured 48 hrs after swim stress and 24 hrs after escape testing. Additionally, when the GRK3 protein levels in stressed rats were grouped based on the rat's escape behavior and compared, there were no differences observed between the groups. (156). This might mean that the single forced swim stress did not affect the levels of GRK3 and that the escape deficits induced were not associated with any change in GRK3. However, the recent studies from our lab observed that in the rats showing LH behavior after being exposed to tail shock stress, there was a greater decrease in GRK3 in the LC 1 hr, 24 hr as well as 48 hr after the tail shock stress (187) than observed after a single swim stress (% vs. % decrease). In the present study brains were isolated 24 hrs after the escape testing (48 hr after the single

swim stress). Therefore, it also is possible that after exposure to a single swim stress, changes in the level of GRK3 in LC that might have been present at the time of escape testing (24hr post-stress) returned to control levels by 48 hr post-stress. The differential effects on levels of GRK3 between the two studies may be due to the method of stressor used, electric shock versus swim stress. Electric shock is considered to be a much more powerful stressor that reportedly causes more marked changes in neurochemistry compared to swim stress (188). In contrast, a single swim stress offers a milder stress paradigm where the effects might not be as sizable or as long lasting (188). This contention is supported by the fact that a single eight-minute swim session was reported to cause an increase in only NE in the medial prefrontal cortex, which is a terminal region of LC projection. This single swim session did not cause any change in other catecholamines (dopamine and serotonin) (188). However, a single electric shock stress session which lasted from 30 to 120 min caused an increase in NE, dopamine and serotonin, indicating a more robust effect (188). Moreover, the electric shock stress paradigm's robustness was underlined by the fact that a single 100 tail shock session caused an increased escape deficits in the total stressed group (unpublished results) (187). In contrast, a single 10 min swim stress did not reveal the presence of any escape deficits when the stressed data was considered collectively. Only after cluster analysis were two clusters identified out of which one cluster showed significant escape deficits compared to

controls. This points to the possibility that the intensity of the single swim stressor was not high enough for the changes to last until the time that the brains were isolated 48 hr later. Future investigations should determine the levels of these proteins in LC at 24 hr post stress, when behavioral changes were observed. Also earlier time points need to be examined to elucidate any temporal changes in GRK3 that might take place and might be responsible for the changes in behavior on exposure to single forced swim stress.

On the other hand, of repeated forced swim stress for three days was associated with decreased levels of GRK3 in the LC of the rats exposed to forced swim stress compared to the control rats. Also, when the GRK3 protein levels in stressed rats were grouped based on the rat's escape behavior, there was a decrease in GRK3 in the LH rats compared to controls or NH rats. This was accompanied by more pronounced escape deficits in the rats showing LH behavior after exposure to the repeated vs. single swim stress, as was discussed earlier. Thus only the behavioral changes induced by three days of repeated stress were associated with changes in the level of GRK3.

The central question that can be raised is why were levels of GRK3 reduced? Reports from our lab and many others can provide insights into this. It was demonstrated using neuroblastoma BE(2)-C cells that binding of heat-shock protein 90 (Hsp90) to GRK3 inhibited GRK3 degradation (35). Further, an inhibitor of HSP90, geldanamycin, abolished binding of Hsp90 with GRK3,

leading to proteosomal degradation of GRK3 and rapid and marked reduction of GKR3 levels in the cells (35). Further, calcium activated calpains increased GRK3 degradation and a calpain inhibitor prevented this enhanced degradation of GRK3 (35). In addition, there are reports that during conditions of continuous neuronal firing, as noted with seizures, there are huge accumulations of intracellular calcium (191). Thus, disruption of the interaction of Hsp90 with GRK3 during exposure to repeated stress might play a role in enhanced degradation of GRK3. Moreover, it can be posited that increased neuronal firing during repeated exposure to stress might lead to increased intracellular calcium, promoting degradation of GRK3.

The suggested inverse relationship between GRK3 levels in LC and an increased propensity for the adverse consequences of stress is not universally supported in the literature. Studies examining the effects of stress have been conducted in GRK3 knockout mice. The mice deficient in GRK3 exhibit decreased immobility as compared to the wild type when subjected to a single forced swim stress. This result was interpreted as indicative of decreased behavioral despair. It was suggested that elimination of GRK3 guards against the adverse effects of stress (151, 152), a conclusion directly the opposite to the hypothesized role for GRK3 in LC in this study. However, it is important to remember that loss of GRK3 is global in knockout mice. There are reports suggesting that while GRK3 turns off the signaling of various receptors in some

brain regions, GRK3 enables signaling of receptors in other brain regions associated with stress. For example, studies show that dynorphin, acting via kappa opioid receptors in nucleus accumbens can contribute to aversive behavior in response to stress. This effect is mediated by GRK3-induced phosphorylation of kappa opioid receptors and subsequent stimulation of p38 MAPK (41, 150-153). Thus the studies involving GRK3 knockout mice involve global elimination of the role of GRK3 rather than the effect of diminution of the role of GRK3 in a specific brain region and therefore may have very different overall effects on the behavior of the animal.

In contrast to the changes in levels of GRK3, the levels of another GRK from the same family as GRK3, namely GRK2, in LC of the rats were similar across the groups after exposure to both single and repeated forced swim stress. Studies have observed lower levels of membrane GRK3, without any change in levels or mRNA of GRK2, in the cortex of patients diagnosed with bipolar disorder compared to the controls (46, 47). Also, long-term treatment with therapeutically relevant doses of the mood-stabilizers lithium and carbamazepine resulted in increased membrane GRK3 but not GRK2 in the frontal cortex of rats (47). Although the role of GRK2 cannot be completely discounted, these studies may indicate a comparatively more significant role for GRK3 in stress-associated affective disorders compared to the role of GRK2 in discrete brain regions.

In light of the changes observed in levels of GRK3 associated with forced swim stress, the next question that arises is how the receptors regulated by GRK3 are affected by forced swim stress?

5.4. Repeated forced swim stress induced changes in levels of CRF_1-R.

The fact that corticotrophin releasing factor (CRF) neurotransmission in LC is involved during stress has been reported. CRF-like immunoreactivity was almost doubled in LC or rats exposed to acute immobilization stress as well as rats exposed to chronic variable unpredictable stressors (192). Also in LC neurons, there is an increase in the ratio of cytoplasmic to total CRF_1-R 1 hr after exposure to a single forced swim. The ratio of cytoplasmic to total CRF1-R was increased further at 24 hr after the single swim stress (193). This study suggested that CRF discharged into LC after exposure to swim stress acted on the CRF_1-R on LC neurons and there was subsequent internalization of the receptors due to which there was increase in the ratio of cytoplasmic to total CRF_1-R. It needs to be noted here that an increase in ratio of cytoplasmic to total receptors does not necessarily mean a decrease in the total receptor levels. Given the fact that there were no changes in levels of GRK3 in the present study with single forced swim stress, it could be expected that the levels of CRF_1-R might go down in LC of rats exposed to single forced swim stress. However, in

the present study, no changes in the levels of CRF_1-R were observed in LC of rats exposed to single forced swim stress compared to the control. Similarly, when the levels of CRF_1-R were compared between LH, NH and control rats there were no differences. In view of the other study mentioned just above, the most likely explanation is that in the present study although there might also have been internalization of the CRF_1-R, there was no change in total number of the receptors.

Other possible explanations might explain the lack of change in CRF_1-R after single swim stress, especially considering that there were two behaviorally different clusters of animals after exposure to a single swim stress. Reports from our lab indicated a decrease in the levels of GRK3 in LC of rats that demonstrated learn-helplessness after being exposed to single 100 min session of tail shock stress. This decrease was accompanied by differential changes in the levels of α_{2A}-AR and CRF_1-R in LC of the LH (no change) and NH rats (decrease) (156). It is possible that since the brains were isolated 48 hr after the last swim, any change in level of the receptors that might have occurred after the single swim stress, have returned to the normal levels.

With regard to the findings in the present study it might be relevant to note the previous reports that a single 15 min continuous forced swim stress resulted in increased responses to low doses of CRF accompanied by a decrease in the maximal response compared to the non-swim controls (132). The lack of

differences in the level of GRK3 or the CRF_1-R in the present study does not directly explain these electrophysiological findings. However, under the circumstances of unchanged numbers of CRF_1-R in the LC neurons, one explanation could be an increased receptor-effector coupling after exposure to single swim stress. There is evidence in literature for this explanation. There was enhanced CRF_1-R coupling to G_s-protein, as indicated by GTPγS binding in LC of male rats, after exposure to swim stress compared to that of the unstressed rats (133). Also in the LC of male rats exposed to stress an augmented cAMP mediated response to CRF compared to unstressed rats was observed (133). Thus it is possible that the rats that demonstrated behavioral deficits had an increased coupling of the CRF_1-R to the effectors in the signal cascade and this contributed to an enhanced CRF_1-R signaling. Alternatively, the augmented response to CRF reported after a single swim stress may reflect elevated levels of endogenous CRF. Under these circumstances, the response to lower concentrations of exogenous CRF might be enhanced. However the maximum response to exogenous CRF would be reduced. The possible confounding influence of endogenous CRF is a disadvantage of examining dose-response curves *in vivo* in brain regions.

In contrast to the single swim stress, there was increased in CRF_1-R in rats that were LH after being exposed to repeated forced swim stress. These LH rats also had a decreased in GRK3 in LC, as discussed in the previous section.

A previous study from our lab observed that repeated exposure to tail shock was associated with decreased GRK3 and increased CRF_1-R in rats that developed LH behavior. GRK3 preferentially regulates CRF_1-R (31-33, 37). Therefore, lowering GRK3 would likely contribute to CRF_1-R up-regulation in LC during stress. Reports from our lab indicated that activation of CRF_1-R by CRF and α_{2A}-AR by epinephrine cause translocation of transcription factors, Sp-1 and Ap-2, and also increased their levels in the nucleus via mechanisms involving extracellular signal-regulated kinase (ERK) 1/2 (147, 148). Therefore it is possible that under conditions of decreased GRK3 and increased receptor signaling there can be increased transcription of messenger RNA of CRF_1-R and α_{2A}-AR occurring via activation of ERK mediated pathways leading to a subsequent increase in levels of these receptors. But how do the changes in proteins relate to the changes in behavior upon exposure to stress?

GRK3 contributes to termination of receptor signaling by phosphorylating agonist-occupied receptors. Receptor phosphorylation promotes recruitment of arrestin and initiates the process of desensitization. This process leads to curtailment of the signaling of the receptors in the face of elevated agonist concentration during exposure to stress. Therefore, a decrease in GRK3 in LC neurons should increase the responsiveness of CRF_1-R to CRF during exposure to stress. This would contribute to increased activation of the LC noradrenergic neurons by CRF. These neurons have been suggested to mediate the adverse

consequences of exposure to repeated stress (27). In addition, the fact that there were changes in GRK3 and CRF_1-R in LH animals exposed to repeated forced swim stress reinforces the notion of the more robust effects of repeated stress compared to the single stress.

To elicit changes in behavior the sensitivity of the receptors needs to change. Numerous studies in the literature have observed changes in LC neuronal activities on exposure to repeated and single stress and provide insight into the physiological changes that might be responsible for more robust effects of repeated stress on the behavior. The effect of single and repeated stress sessions (electric shock) on LC discharge rate in response to intra-cerebroventricular administration of CRF has been studied previously. After a single stress exposure, the sensitivity of LC neurons to exogenous CRF was attenuated (53). This is contrary to what would be expected from the data from a previous study in our lab where exposure to a single tail shock stress session produced a decrease in GRK3 and no change in CRF_1-R in LH rats (187). This might happen if there is a compensatory increase in another mechanism of CRF_1-R signal termination like regulator of g protein signaling (RGS) during exposure to stress or a decrease in coupling of CRF_1-R to the ion channels through which it mediates its action (194) . In contrast to the inescapable shock, there was an increase in response to CRF after exposure to single swim stress (132). Thus the differential change in response to CRF after exposure to single

stress might be attributed to the variable underlying changes in neurochemistry due to different methods of stress exposure. However, after exposure to the repeated electric footshock stress sessions, there was a decrease in the maximum response but an increase in response to lower doses of CRF compared to the controls or rats exposed to a single stress (53). The response of CRF seen above was by using exogenous CRF. However, the authors also studied the response to a hypotensive challenge that increases release of endogenous CRF within LC. This manipulation caused an elevation of LC discharge and the response was greatly decreased after a single stress session. The response was restored after repeated stress sessions, implying a decreased sensitivity to CRF after a single stress and an increased sensitivity to CRF after repeated stress sessions (53). These stress-initiated increases in the sensitivity of LC neurons to CRF after repeated stress may result in stimulation of LC neurons by lowering the minimally effective dose of CRF in LC. Decreases in GRK3 in LC on exposure to repeated stress also should impair the regulation of the CRF1-R and increase the response of the LC to endogenous CRF. This impaired regulation could contribute to hyper-activation of the CRF-LC-NE feed-forward circuit leading to NE activation of CRF neurons that project back to LC. This would augment the adverse behavioral consequences like hyper-arousal and difficulties in concentration that are associated with the negative consequences of repeated stress (21).

5.5 Repeated forced swim stress induced changes in levels of α_{2A}-AR.

In the present study, there were similar levels of α_{2A}-AR in the LC of rats exposed to single forced swim stress and the control rats (Fig 8). Even when the levels of the α_{2A}-AR were observed in the LH and NH rats, there were no differences. This was different from the results after a single tail shock stress session. After a single tail shock stress session, although there was no difference in α_{2A}-AR in the LC of LH rats, the α_{2A}-AR in the NH rats were decreased. It might be argued that if there was no change in GRK3 levels in the LC on exposure to a single swim stress, any change in α_{2A}-AR levels that might have taken place due to exposure to stress might have come back to normal by the time the brain was isolated (48 hrs post stress). Since there were no differences in the level of α_{2A}-AR in LC of rats between any groups, it is possible that α_{2A}-AR have no role to play in the development of single swim stress-induced LH behavior. However, there is considerable evidence for role for α_{2A}-AR in stress induced behavioral changes. This evidence suggests a significant variability in results depending upon the nature or duration of the stress and the time interval between the stress and when the brain was removed from the rat. Changes in the level of α_{2A}-AR are reported to be related to the method and duration of the stress (145). For example, time- and stress-dependent elevation

(1hr of cold stress) or lowering (4hr of cold stress; 1hr and 4 hr of restraint stress) of levels of α_{2A}-AR have been reported in rat brain stem (145).

Increases in levels of α_{2A}-AR in the LC of rats exposed to repeated forced swim stress were noted in this study. When the levels of α_{2A}-AR in LC of the rats exposed to repeated forced swim stress were segregated based on the escape behavior, there was an increase in α_{2A}-AR in the LH rats. These increases in α_{2A}-AR levels can be attributed to the decrease in GRK3 in LC of rats exposed to repeated forced swim stress. One important limitation in the present study was that the levels of α_{2A}-AR measured in LC were the total α_{2A}-AR present, including those pre- and post-synaptically located on the neurons as well as those on the glia. Therefore it is not possible to differentiate the changes specific to each of these locations, which could help understand the roles played by specifically located α_{2A}-AR during stress. However, the observations in the present study are in agreement with previous studies from our lab where there was an increase in CRF_1-R in LC of LH rats exposed to repeated tail shock stress sessions (187). Future investigations should determine the levels of these proteins in LC at 24hr post stress, where behavioral changes were observed, as well as earlier time points to elucidate the temporal changes in protein responsible for the changes in behavior on exposure to single forced swim stress. Identifying the specific changes in pre-synaptic, post-synaptic and glial α_{2A}-AR will help elucidate the exact role played by these receptors during exposure to stress. Such studies

might be feasible using immunohistochemical, electron microscopy or autoradiographic techniques, but only semi-quantitative information could be obtained. In addition the amount of tissue obtained from each rats would make such studies challenging.

5.6. Repeated forced swim stress increases response to NE of LC neurons in rat brain stem slices.

Increased response to low concentrations of NE in the LC neurons of rats exposed to repeated forced swim stress compared to the control rats was observed in this study. The maximal responses to NE were similar across the groups. The preferential α_1-AR antagonist prazosin (100 nM) was present in these experiments to minimize the possible influence of α_1-AR on the responses to NE observed. Regarding the responses to the maximal concentrations of NE, we cannot rule out the possibility of involvement of other actions of NE in addition to action on α_2-AR. Since 100 nM prazosin is not a saturating concentration, a contribution of additional action of NE on α_1-AR cannot be ruled out at higher NE concentrations. However, higher concentrations of prazosin resulted in α_2-AR blockade, making the use of these higher concentrations untenable.

The increase in response at the lower concentrations of NE suggests increased responsiveness of α_{2A}-AR to NE. These increases in responses are

important given the fact that they occur at lower doses that are more relevant physiologically. Although there can be other explanations, considered in total, the most probable explanation for these observations is that the increase in responses to NE in the repeated swim stressed rats was due to increased levels of α_{2A}-AR and decreased GRK3 in the LC of the repeated forced swim rats compared to the controls. Imagine a situation where, for example, there are 1000 receptors but only 500 are needed to achieve maximum response. Therefore, 250 receptors will be occupied at the EC_{50}, or 25% of the total receptors. If there is a 32% increase in receptor expression, the total is now 1320 receptors. However only 250 receptors are required to achieve half-maximal response, or 18% of the increased total receptor number. Therefore, half-maximal response would be observed at the NE concentration that occupies 18% rather than 25% of the receptors. In addition, the maximal response would be observed at concentrations of NE that occupy 38% of the receptors. However, the same maximal response would be observed. In keeping with this explanation, the LC has been found to have a high reserve pool of α_{2A}-AR and the receptors are quickly turned over (195). Thus, repeated forced swim stress accompanied by a decrease in GRK3 and an increase in α_{2A}-AR was likely responsible for the increase in response to lower concentrations of NE without any increase in maximal response.

There are several other possible explanations for the greater responses to NE. First there might be an increased coupling of the α_{2A}-AR to its g protein and other mediators of the signaling cascade (cellular effectors) or an increased g protein-induced activation of the potassium channels that are activated by agonist occupied α_{2A}-AR. Under these circumstances if the levels of the total number of potassium channels remained unchanged, there will be an increased response at the lower doses while saturating the signaling machinery at the higher doses thus preventing an increase in the maximal response.

The observed increase in response of the α_{2A}-AR is in agreement with various observations found in the literature. It is reported that there was an augmentation of sensitivity to suppression of LC firing by cumulative doses of clonidine after chronic immobilization stress in rats (52). Also, male tree rats show increased α_{2A}-AR in brain after being repeatedly stressed (196). Additionally, increased α_2-adrenoceptor agonist UK14304 potency to stimulate $GTP_{\gamma S}$ binding has been reported in brains of depressed human subjects suggesting an increase in receptor-$G\alpha_{i/o}$-protein coupling (197). Finally, it was reported that in cases of suicide patients suffering from major depression, α_{2a}-AR density was elevated in frontal cortex, hypothalamus and LC. This increase was observed in binding studies involving [^{3}H] agonist (79, 198, 199). However, the increase was not found when [^{3}H] antagonists were used in the binding studies (200-202). This suggests a selective augmentation in the high-affinity G-protein

coupled state of α_{2a}-AR in brain of patients suffering from major depressive disorders. Thus it is possible that after exposure to repeated stress, the increase in response to α_{2A}-AR might be due to an increase in coupling of α_{2A}-AR to its g protein in the LC neurons, as well as an increase in the number of receptors.

The electrophysiological recordings in the present study were done on the neurons of LC and hence the effects of NE that were observed, were mediated by the post-synaptic α_{2A}-ARs located on the soma. However, it needs to be noted that in LC, α_{2A}-AR also are located presynaptically on afferent nerve terminals and on recurrent collateral projections (29, 203). The NE acting via post-synaptic α_{2A}-AR decreases LC neuronal firing (139, 204). Presynaptically, α_{2A}-AR have been found on noradrenergic afferent terminals to LC as well as non-catecholaminergic terminals that provide innervations of excitatory inputs like glutamergic to the LC (203). These presynaptic α_{2A}-AR on noradrenergic terminals serve as auto-receptors and upon activation inhibit further release of NE onto post-synaptic α_{2A}-AR. Therefore, pre- and post-synaptic α_{2A}-AR would be predicted to have opposite effects on LC firing rates. To determine the overall effects of α_{2A}-AR in LC, consideration should be given to both the presynaptic as well as the postsynaptic receptors.

5.7 Repeated forced swim stress-induced increases TH in LC of rats.

Levels of TH were similar in LC of rats exposed to single forced swim stress compared to the controls. Tyrosine hydroxylase catalyzes the rate-limiting step in NE synthesis in neurons. The TH levels in the LC of the LH rats after a single force swim also were similar to those in the NH as well as in the control rats. It is possible that any change in TH presents 24h after stress, during the escape testing, could have come back to normal 48 hr post stress. Although there was no change in levels of TH, this cannot be considered as indicating lack of any role played by TH during exposure to the single swim stress. It has been suggested that in central nervous system, TH, is in a fractionally subdued condition. The enzymatic function of TH may be elevated under conditions where catecholamine levels in tissues are diminished (205, 206). This happens within a short time frame. The elevations of enzymatic function of TH because of enhanced tissue levels of the enzyme generally involve a longer time frame (207, 208). This can be provided by exposure to repeated stress. Repeated forced swim stress rats showed an increase in the levels of TH in LC. There also was an increase in TH in the LC of LH rats exposed to repeated forced swim stress. This is consistent with various studies where an increase in TH protein and mRNA was observed in LC and its terminal fields in rats exposed to repeated daily stress (209-213). Repeated stress has been associated with the elevations

of neuronal activity of the LC–noradrenergic system on exposure to subsequent stressors leading to elevations of NE levels and discharge in the LC and its projection areas (214). This leads to elevated need for NE biosynthesis after exposure to stress, to replete the stores. The consequences of this enhanced activity are suggested to be increased levels of TH protein and mRNA in LC and the areas where it projects as well as enhanced enzymatic activity of the TH to replete the levels of the catecholamine. Other evidence supports the finding of the present study. Elevated levels of tyrosine hydroxylase in LC have been observed after exposure to repeated intermittent foot shock and noise stress. This effect was attributed to an action of endogenous CRF on LC neurons. A CRF receptor antagonist prevented the effect (215). Since CRF increases the firing of LC neurons, this result is in agreement with the idea of an overall increase in LC activity contributing to increased TH expression.

The clinical data available regarding depressive behavior and TH also are in agreement with observations in animal studies after stress-induced depression-like behavior. Increased levels of TH, as high as 172% of the matched controls, have been observed in LC of patients suffering from depression and from suicide patients (79).

5.8. How do the biochemical changes in the LC of the rats exposed to the repeated forced swim stress contribute to changes in behavior?

The possibility of diminished GRK3 playing a role in stress-related disorders can be argued where a decreased GRK3 increases responsiveness of GPCRs and results in dysregulation of signaling of CRF_1-R and α_{2A}-ARs. This possibly caused a net bias towards excitatory CRF_1-R, leading to hyperactivation of the CRF-LC-NE feed-forward system mentioned above and causing subsequent behavioral modifications associated with the adverse consequences of stress.

Taken collectively, the results in the present study do not explain how the previously reported overall increase in neuronal discharge of LC neurons is achieved after exposure to repeated stress. How could reduced levels of GRK3 and increase in numbers of both CRF_1-R and α_{2A}-AR contribute to this? Stress has been associated with an increase in both CRF and NE in LC. A possible explanation might be that a decrease in GRK3 will cause a more robust increase in responsiveness of the excitatory CRF_1-R as compared to the inhibitory α_{2A}-AR. Some insights into how this might come about are provided by considering the concentration and time of exposure required for desensitization of the respective receptors.

Evidence suggests that CRF_1-R desensitization requires much lower concentrations and a shorter time exposure to CRF (33, 37). However a much higher concentration and more prolonged exposure time of NE (30μM /16-24 hours, is required to desensitize α_{2A}-AR (31). Thus CRF_1-R is more sensitive to agonist-induced desensitization, compared to α_{2A}-AR, and therefore a decrease in GRK3 would be expected to affect the CRF_1-R more. In combination, this would shift the signaling balance in favor of the excitatory CRF_1-R signaling.

Another possible explanation for an increased excitatory influence in LC after repeated forced swim might be the differential signaling pathways of the α_{2A}-AR and CRF_1-R that alter LC firing. The actions of the α_{2A}-AR are often mediated via $G\alpha_{i/o}$ protein-induced suppression of adenylyl cyclase with subsequent reduction of cAMP production (140). However, in LC the inhibitory action on firing of neurons is mediated by direct G protein activation of inward rectifying potassium channels (GIRKs). These GIRKs, upon activation enhance potassium conductance and subsequent hyperpolarization of the neurons (141). In contrast, the action of CRF in LC is mediated by CRF_1-R, which predominantly binds to the guanosine triphosphate (GTP) binding protein, $G\alpha_s$-protein, resulting in increased cyclic adenosine monophosphate (cAMP) production via adenylyl cyclase (129, 130). Evidence suggests the involvement of cAMP in mediating the excitatory actions of CRF in LC (30). The study suggested that corticotrophin releasing factor-induced activation of LC neurons

is primarily via inhibition of a potassium conductance (30). Thus there is a possibility that on exposure to repeated stress and under conditions of increase of NE and CRF, the action of CRF overwhelms the actions of NE, thus leading to an overall increase in firing of LC neurons. When viewed along with the changes in CRF_1-R, it can be postulated that an additional increase in presynaptic α_{2A}-ARs, if it were to occur, would tend to reduce further release of NE and thus further potentiate the excitatory action of CRF. A variation of this alternative explanation is that the opposing effects of enhanced presynaptic and postsynaptic α_{2A}-AR could cancel each other out. Under these circumstances, the end result might be a predominant and enhanced excitatory effect of CRF_1-R. Future studies examining the interaction of the CRF_1-R and α_{2A}-AR on single LC neurons would help clarify these issues.

One area of localization of α_{2A}-AR in LC is on the glial cells adjacent to the TH-positive dendrites (203). This suggests that astrocytes might be a destination for action of catecholamines released into the LC. There is evidence in the literature suggesting that α_{2A}-ARs present on cultured type 1 astroglia from cerebellum elevate the intracellular calcium concentration and those present in glial cell cultures cause accumulation of cAMP (216, 217). The activation of these signaling cascades can modulate changes in the morphological structures of the astrocytes (218). With such morphological changes, one could observe either infusion or retraction of the processes of astrocytes into the dendritic spaces of

LC neurons contributing to a potential regulation of signaling between the neurons in LC. Nothing in the present study addresses this possibility.

5.9 Biochemical parameters can be a good predictor of the escape behavior of rats after exposure to repeated forced swim stress.

The biochemical data for levels of GRK3, CRF_1-R and α_{2A}-AR in LC were collectively subjected to cluster analysis. Two different clusters were identified. When these clusters were matched for symmetry with the clusters obtained from escape behavior data of repeated forced swim stress rats, there was 90% symmetry between the clusters identified from biochemistry and those from behavior. This indicated that GRK3, CRF_1-R and α_{2A}-AR in LC collectively can be used predict the behavior of rat on exposure to repeated forced swim stress.

5.10 Repeated forced swim stress did not induce changes in behavior or levels of GRK3, α_{2A} -AR and CRF_1-R in LC of the rats pretreated with desipramine.

In order to test the hypothesis that pretreatment with an antidepressant drug would prevent the forced swim stress-induced behavioral impairment and accompanied changes in GRK3 and receptors, some rats were treated with DMI,

using a dose previously found to be effective in rats. Pretreatment of the rats with DMI, a tricyclic antidepressant drug, resulted in a significant decrease in immobility time in the repeated forced swim stress, as compared to the non-drug treated rats, and prevented the progressive increase in immobility. DMI also increased the time spent in climbing behavior on all three days. DMI is a potent NE reuptake inhibitor. In the forced swim paradigm the active behavior is comprised of climbing and swimming. Inhibitors of noradrenergic reuptake preferentially alter the climbing parameter.

DMI pre-treatment prevented LH behavior induced by repeated forced swim stress. This was in agreement with another study where DMI treatment caused a significant decrease in stress-induced escape failures (219). The point of difference with that study was that the authors used inescapable shock as opposed to forced swim stress used in the present study. Thus DMI prevents the repeated stress-induced adverse behavioral changes regardless of the inescapable stress employed to reveal the stress susceptibility.

DMI diminishes the basal discharge activities of LC neurons and diminishes sensory–evoked discharge of LC neurons via a mechanism involving α_{2A}-AR (220-222). DMI also increases extracellular NE by 96% and thus prevents decline in intra-synaptic NE concentrations during repeated stress (29). Evidence indicates a role of LC in mediating the antidepressive effects of DMI as only destruction of LC, but not the destruction of ventral noradrenergic neurons,

altered the effects of DMI (126). In the present study, on pre-treating the rats with DMI and then exposing them to repeated forced swim stress, there was no change in levels of GRK3 in LC. The inhibitory effect of DMI treatment on LC firing would be expected to reduce or prevent any degradation of GRK3 mediated via increased intracellular calcium associated with increased neuronal firing of LC during stress. Clinical and animal studies have indicated that CRF function in LC is elevated during stress inducing adverse consequences, like depression (223). DMI treatment was found to blunt the CRF discharge into LC during stress and will blunt the stress induced increase in LC activities. This could in turn prevent the adverse consequences of repeated stress, for example, the escape deficits.

In the present study, on pre-treating the rats with DMI and then exposing them to repeated forced swim stress, there was no change in levels of CRF_1-R and α_{2A}-AR in LC of the rats. It must be noted that the focus of the present study was to observe the effects of DMI treatment to prevent repeated forced swim stress-induced changes in the receptor levels rather than compare the effects of DMI treatment vs. no treatment on these receptors. However, previous study from our lab indicate that there was no effect of 14 days of treatment of DMI (5mg/kg; bid; Intraperitoneal) on the locus coeruleus levels of GRK3, GRK2, TH, CRF_1-R and α_{2A}-AR in control rats compared to the non-drug treated control rats (unpublished data) (187). It is possible that prevention of changes in receptors and their sensitivities prevented the dysregulation (hyperactivation) of the CRF -

LC - noradrenergic system that has been purported to cause the adverse behavioral consequences of stress. However, there is a possibility that repeated forced swim stress in the DMI-treated rats might cause down-regulation of the CRF_1-R and α_{2A}-AR in LC of rats, leading to the normal levels and functioning of GRK3 observed with DMI treatment.

DMI, as a NE reuptake inhibitor, will increase the synaptic concentrations of NE, which in turn will increase the chances of desensitization of α_{2A}-AR (224). However, the focus of this study was to observe the effects of DMI in the repeated forced swim stress animals. Thus, this present study demonstrates pretreatment with DMI abolished any repeated forced swim stress–induced elevation in α_{2A}-AR and CRF_1-R levels.

Previous studies have provided evidence that treatment with DMI significantly diminish TH levels (225, 226). The present study and many other studies have demonstrated that there is a stress-induced elevation of TH in LC of rats and human (79). Therefore it can be anticipated that use of antidepressant drugs would reduce TH in LC or at least abolish of the increase in TH on exposure to stress. In the present study DMI pretreatment prevented of the repeated forced swim stress-induced upregulation of TH. This was observed from the comparison of TH levels in LC of DMI-treated non-swim control and DMI-treated repeated forced swim stressed rats. The results suggest that DMI prevents the repeated stress induced increase in LC noradrenergic activities

leading to a subsequent blocking of the increase in TH and the escape deficits. Thus DMI pre-treatment prevents the forced swim stress-induced increase in activities of LC neurons, which prevents the change in TH levels as well as the stress-induced decrease in GRK3. This will help keep the regulation of receptors at a normal level, which in turn will block the adverse behavioral consequences of stress.

6. SUMMARY AND CONCLUSION

1. Single and repeated forced swim stress leads to bifurcation of the stressed rats into two significantly different clusters. One of the clusters showed significant behavioral deficits in an escape task compared to the control groups and is called LH. The other cluster demonstrated escape behavior similar to the control groups and is called NH. The impairment of escape behavior in the animals exposed to repeated stress was greater compared to the impairment after a single stress. Thus, a new model for causing LH was validated in this study and demonstrated that electrical shock paradigms are not the only means to cause LH behavior. Hence, forced swim stress can be used as an inescapable stress paradigm to identify stress-susceptible and stress-resilient populations in rats.
2. Repeated daily forced swim stress also caused a progressive increase in immobility during the swim from day 1 to day 3.
3. Exposure to repeated forced swim stress decreased levels of GRK3 and increased TH, α_{2A}-AR and CRF_1-R in LC of LH, but not NH rats. No biochemical changes were observed after single forced swim stress.
4. Exposure to repeated forced swim stress also was associated with an increase in responses to lower doses NE in LC neurons measured in brain

stem slices. This is likely attributable to the increase in α_{2A}-AR numbers and reduced levels of GRK3 in LC neurons after repeated forced swim stress.

5. Repeated forced swim stress did not induce increases in immobility and escape deficits in the shuttle-box in the rats pretreated with DMI. Moreover, repeated forced swim stress did not cause any decrease in GRK3 or increases in CRF_1-R and α_{2A}-AR in the LC of rats pretreated with DMI. DMI increases availability of NE, resulting in an inhibitory tone on the LC neurons and abolition of the hyperactivity of LC neurons on exposure to repeated forced swim stress, thus preventing the associated biochemical and behavioral changes.
6. Repeated forced swim stress can be used to study the behavioral changes and the accompanying underlying neurobiological changes in stress-susceptibility and stress-resilience. This model of repeated forced swim stress also can be used to predict actions of drugs that can prevent stress-induced induction of LH.
7. Additional studies will be needed to establish a cause and effect relationship between a decrease in GRK3 in LC and the repeated forced swim stress-induced behavioral deficits and the enhanced electrophysiological response of LC neurons to α_{2A}-AR activation induced.

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