"To evaluate effect of *Hypericum perforatum* on chronic stress induced alterations in social behavior"

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CERTIFICATE

This is to certify that the project report entitled "**To evaluate effect of *Hypericum perforatum* on chronic stress induced alterations in social behavior**", submitted by Aarushi Sharma (111561) in partial fulfillment for the award of degree of Bachelor of Technology in Biotechnology to Jaypee University of Information Technology, Waknaghat, Solan has been carried out under my supervision.

This work has not been submitted partially or fully to any other University or institute for the award of this or any other degree or diploma.

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SUMMARY

Stress is a normal physical response to events that make you feel threatened or upset your balance in some way. It is the response of the body to demands made upon it. It is the result of our reaction to outside events, not necessarily the events themselves.

Modern life is full of hassles, deadlines, frustrations, and demands and in today's time, there's hardly anyone who doesn't suffer from stress in one form or the other. Stress can affect various aspects of life - the physical & physiological functioning, psychological & mental well-being, social life & relationships, etc.; as a result, it becomes a necessity to understand the underlying causes of stress, its effect on our mind and body and also know what can be done to avoid it.

In the present study, we have used rat and mice (Swiss albino) models to understand how stress, be it acute or chronic, affects the social behavior. Initially, four groups of rats were taken and were subjected to acute stress in the form of temporary restrain. Their social behavior was observed by means of the three-chamber paradigm test known as 'Crawley's sociability and preference for social novelty' protocol. Following this study, another four groups of mice were taken and subjected to chronic unpredictable mild stress (CUMS) and their social behavior was also observed by means of a three-chambered apparatus.

Aarushi Sharma

Dr. M. Udayabanu

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LIST OF ABBREVIATIONS

CUMS	Chronic Unpredictable Mild Stress
CRF	Corticotropin-Releasing Factor
ACTH	Adrenocorticotropic Hormone
LHPA axis	Limbic–Hypothalamic–Pituitary–Adrenal axis
HPA axis	Hypothalamic–Pituitary–Adrenal axis
GAS	General Adaptation Syndrome
SJW	St. John's Wort
SSRI	Selective Serotonin Reuptake Inhibitors
PMDD	Premenstrual Dysphoric Disorder

CHAPTER 1

INTRODUCTION

In order to understand the need for this study, it's important to have a basic understanding of stress, importance of social behavior and how it is affected by stress. It's also important to be clear about the models that have been used.

1.1) STRESS AND ITS TYPES

Everyone needs a little stress in their lives to perform. Think of a musical instrument, without enough tension, it would go off tune but too much stress or tension could result in the string to snap. It's the same thing with our mind and body; the trick is to keep stress under control and in balance.

Stress starts off as a feeling and a reaction to certain situations; it's the body's way of making you more alert and ready to take on a challenge. We might think that the stress happens only in the mind; however, research has shown that the entire body can be affected by stress. (1)

When we encounter a perceived threat or are placed in a stressful situation, the hypothalamus, a tiny region at the base of your brain, sets of an alarm system in your body through a combination of nerve and hormonal signals. This system prompts your adrenal glands, located on top of your kidneys to release a surge of hormones including adrenaline and cortisol. Adrenaline increases your heart rate, increases your blood pressure and boosts energy supplies. Cortisol, the primary stress hormone, increases sugars, glucose in the bloodstream; this natural reaction is called the **stress response** and it can help us out a lot when we are in a critical situation. (2, 3)

All of these stress reactions enhance a person's ability to perform well under pressure. In a milder form and if managed, the stress response can help you concentrate and perform better. The problem is that the body doesn't always reset itself to normal and in the long term, if your mind and body are constantly on edge, you may face health problems. Too much stress can bring on a number of health problems or increase the severity of them like diabetes and heart disease. Long term stress can also disrupt brain structure and function. (4-7)

The good news is that we have a lot of control over stress and if we are able to figure out what stresses us out, we can make a plan to manage stress and keep it working for you.

The different types of stress are as follows:

- **Acute stress**

Acute stress is the most common form of stress. It comes from demands and pressures of the recent past and anticipated demands and pressures of the near future. Acute stress is thrilling

and exciting in small doses, but too much is exhausting. Overdoing on short-term stress can lead to psychological distress, tension headaches, upset stomach and other symptoms.

Fortunately, acute stress symptoms are recognized by most people. It's a laundry list of what has gone awry in their lives.

Because it is short term, acute stress doesn't have enough time to do the extensive damage associated with long-term stress. The most common symptoms are:

- Emotional distress — some combination of anger or irritability, anxiety and depression, the three stress emotions.
- Muscular problems including tension headache, back pain, jaw pain and the muscular tensions that lead to pulled muscles and tendon and ligament problems.
- Stomach, gut and bowel problems such as heartburn, acid stomach, flatulence, diarrhea, constipation and irritable bowel syndrome.
- Transient over arousal leads to elevation in blood pressure, rapid heartbeat, sweaty palms, heart palpitations, dizziness, migraine headaches, cold hands or feet, shortness of breath and chest pain.

Acute stress can crop up in anyone's life, and it is highly treatable and manageable. (8)

- **Episodic stress**

There are those, however, who suffer acute stress frequently, whose lives are so disordered that they are studies in chaos and crisis. They're always in a rush, but always late. If something can go wrong, it does. They take on too much, have too many irons in the fire, and can't organize the slew of self-inflicted demands and pressures clamoring for their attention. They seem perpetually in the clutches of acute stress.

It is common for people with acute stress reactions to be over aroused, short-tempered, irritable, anxious and tense. Often, they describe themselves as having "a lot of nervous energy." Always in a hurry, they tend to be abrupt, and sometimes their irritability comes across as hostility. Interpersonal relationships deteriorate rapidly when others respond with real hostility. The workplace becomes a very stressful place for them.

The cardiac prone, "Type A" personality described by cardiologists is similar to an extreme case of episodic acute stress. Type A's have an "excessive competitive drive, aggressiveness, impatience, and a harrying sense of time urgency." In addition there is a "free-floating, but well-rationalized form of hostility, and almost always a deep-seated insecurity." Such personality characteristics would seem to create frequent episodes of acute stress for the Type A individual. Another form of episodic acute stress comes from ceaseless worry. "Worry

warts" see disaster around every corner and pessimistically forecast catastrophe in every situation. The world is a dangerous, unrewarding, punitive place where something awful is always about to happen. These "awfulizers" also tend to be over aroused and tense, but are more anxious and depressed than angry and hostile.

The symptoms of episodic acute stress are the symptoms of extended over arousal: persistent tension headaches, migraines, hypertension, chest pain and heart disease. Treating episodic acute stress requires intervention on a number of levels, generally requiring professional help, which may take many months. (8)

- **Chronic stress**

While acute stress can be thrilling and exciting, chronic stress is not. This is the grinding stress that wears people away day after day, year after year. Chronic stress destroys bodies, minds and lives. It wreaks havoc through long-term attrition.

Chronic stress comes when a person never sees a way out of a miserable situation. It's the stress of unrelenting demands and pressures for seemingly interminable periods of time. With no hope, the individual gives up searching for solutions.

The worst aspect of chronic stress is that people get used to it. They forget it's there. People are immediately aware of acute stress because it is new; they ignore chronic stress because it is old, familiar, and sometimes, almost comfortable. Because physical and mental resources are depleted through long-term attrition, the symptoms of chronic stress are difficult to treat and may require extended medical as well as behavioral treatment and stress management. (8, 9)

1.2) IMPORTANCE OF SOCIAL BEHAVIOR

Animal behavior is the bridge between the molecular and physiological aspects of biology and the ecological. It is the link between organisms and environment and between the nervous system and the ecosystem, and is one of the most important properties of animal life. Behavior plays a critical role in biological adaptations. Behavior is how we humans define our own lives. It is that part of an organism by which it interacts with its environment. Behavior is as much a part of an organisms as its coat, wings etc. The beauty of an animal includes its behavioral attributes. (10)

For the same reasons that we study the universe and subatomic particles there is intrinsic interest in the study of animals. Research on animal behavior and behavioral ecology has

been burgeoning in recent years despite below inflation increases (and often decreases) in research funding.

- Many problems in human society are often related to the interaction of environment and behavior or genetics and behavior. Increasingly social scientists are turning to animal behavior as a framework in which to interpret human society and to understand possible causes of societal problems. (11)
- The methodology applied to study animal behavior has had a tremendous impact in psychology and the social sciences. Aspects of experimental design, observation techniques, and attention to nonverbal communication signals were often developed in animal behavior studies before their application to studies of human behavior. The behavioral study of humans would be much diminished today without the influence of animal research. (10)
- The comparative study of behavior over a wide range of species can provide insights into influences affecting human behavior. The richness of developmental processes in behavior, including multiple sources and the consequences of experience are significant in understanding processes of human development. (12)
- Research on animals has developed many of the important concepts relating to coping with stress, for example studies of the importance of prediction and control on coping behavior. (13)
- Neuroethology, the integration of animal behavior and the neurosciences, provides important frameworks for hypothesizing neural mechanisms. Careful behavioral data allow neurobiologists to narrow the scope of their studies and to focus on relevant input stimuli and attend to relevant responses. In many case the use of species specific natural stimuli has led to new insights about neural structure and function that contrast with results obtained using non-relevant stimuli. (14)
- Behavioural baseline is a critical determinant of response to drugs and other manipulations. Deficits in social interaction are important early markers for disorders like autism and related neurodevelopmental disorders with strong genetic components. Recent work in animal behavior has demonstrated a downward influence of behavior and social organization on physiological and cellular processes. (15)

  All animals need to interact with others of the same species, even if it is only to mate. To date, social behavior has been studied mainly at two extremes: detailed observation of pairs; and studies of the collective behavior of large groups, such as flocks of birds. However, to gain an understanding of social behavior in mammals will require an approach that falls

between these two extremes. It is necessary to study animals in larger groups, rather than in pairs, and also to track individuals rather than looking at the activity of the group as a whole. (12, 16)

1.3) HOW STRESS GENERALLY AFFECTS SOCIAL BEHAVIOR?

The neurobiology of stress and the neurobiology of social behavior are deeply intertwined. Social interactions serve as an evolutionarily important source of stress, and one that is virtually ubiquitous among mammalian species. Social stress strongly impacts behavior, generally reducing aggression and enhancing defensiveness, both inside and outside the stress situation. (17) The effects of stress exposure and consequent trajectory depend on the nature of the stressor, the severity, duration (acute vs. chronic), sex/gender, genetics, timing of exposure (early life, adolescence, adulthood or aging) as well as the perception of the stressor by the individual–for example, stressor controllability dramatically affects resilience versus vulnerability as an outcome. (18, 19) The social environment interacts with stress on almost every front: social interactions can be potent stressors; they can buffer the response to an external stressor; and social behavior often changes in response to stressful life experience. (18) Social and sexual behaviors may be reduced in subordinate animals, as is activity and responsivity to normally rewarding events. However, some components of these changes may be dependent on the presence of a dominant, rather than representing a longer-term and general alteration in behavior. (20)

1.4) ANIMAL MODELS

Among the many advantages to using the rat/mouse as a model organism, the most important is their striking similarity to humans in anatomy, physiology, and genetics. Over 95% of the mouse genome is similar to our own, making mouse genetic research particularly applicable to human disease.

Practically, mice are a cost-effective and efficient tool to speed research and the development of drug therapies. Mice are small, have a short generation time and an accelerated lifespan (one mouse year equals about 30 human years), keeping the costs, space, and time required to perform research manageable. (21)

Furthermore, while effects of stress on social behavior are evident in humans, most of our understanding of these impacts, and of the underlying molecular and cellular mechanisms, comes from rodent studies. (22) Animal models of social stress are varied, ranging from a focus on acute, intermittent, or chronic exposure involving agonistic behavior to social

isolation. (17) The relative stressfulness of these experiences may depend on the species, sex, and age of the subjects. Social stress models often produce victorious and defeated, or dominant and subordinate, animals that may be compared to each other or to controls, but the appropriateness of specific types of comparisons and the interpretations of their differences may vary for the different models. (22) The current findings indicate that social stress models can provide high magnitude and appropriate stressors for research, but additionally suggest a need for caution in interpretation of the findings of these models and care in analysis of their underlying mechanisms. (17)

1.5) NEED FOR THIS STUDY

Modern life is full of hassles, deadlines, frustrations, and demands and in today's time, there's hardly anyone who doesn't suffer from stress in one form or the other. Stress can affect various aspects of life - the physical & physiological functioning, psychological & mental well-being, social life & relationships, etc.; as a result, it becomes a necessity to understand the underlying causes of stress, its effect on our mind and body and also know what can be done to avoid it.

This study discusses the significance of the stress concept in gaining a better understanding of social mechanisms in nonhuman mammals. The triggers of stress reactions are mainly psychical processes resulting from the assessment of a situation by an individual. Dependent on the coping behavior of the individual, these processes lead to different physiological response patterns, which can result in a number of pathophysiological effects. Social stressors have proven to be potent across a wide range of species, and their study in rodents will lead to a greater understanding of the role of stressor type, timing, and other factors impacting physiology and behavior.

CHAPTER 2

REVIEW OF LITERATURE

2.1) PHYSIOLOGY OF STRESS

Stress arises when individuals perceive that they cannot adequately cope with the demands being made on them or with demands being made on them or with threats to their well-being. (23)

There are three ways by which stress gets under the skin:

1. The Fight or Flight Response

The flight or fight response, also called the "acute stress response" was first described by Walter Cannon in the 1920s as a theory that animals react to threats with a general discharge of the sympathetic nervous system. The response was later recognized as the first stage of a general adaptation syndrome that regulates stress responses among vertebrates and other organisms.

To produce the fight-or-flight response, the hypothalamus activates two systems: the sympathetic nervous system and the adrenal-cortical system. The sympathetic nervous system uses nerve pathways to initiate reactions in the body, and the adrenal-cortical system uses the bloodstream. The combined effects of these two systems are the fight-or-flight response. (24)

When the hypothalamus tells the sympathetic nervous system to kick into gear, the overall effect is that the body speeds up, tenses up and becomes generally very alert. The sympathetic nervous system sends out impulses to glands and smooth muscles and tells the adrenal medulla to release epinephrine (adrenaline) and norepinephrine (noradrenaline) into the bloodstream. These "stress hormones" cause several changes in the body, including an increase in heart rate and blood pressure. (24)

At the same time, the hypothalamus releases corticotropin-releasing factor (CRF) into the pituitary gland, activating the adrenal-cortical system. The pituitary gland (a major endocrine gland) secretes the hormone ACTH (adrenocorticotropic hormone). ACTH moves through the bloodstream and ultimately arrives at the adrenal cortex, where it activates the release of approximately 30 different hormones that get the body prepared to deal with a threat.

The sudden flood of epinephrine, norepinephrine and dozens of other hormones causes changes in the body. (24)

Fear -- and the fight-or-flight response in particular -- is an instinct that every animal possesses.

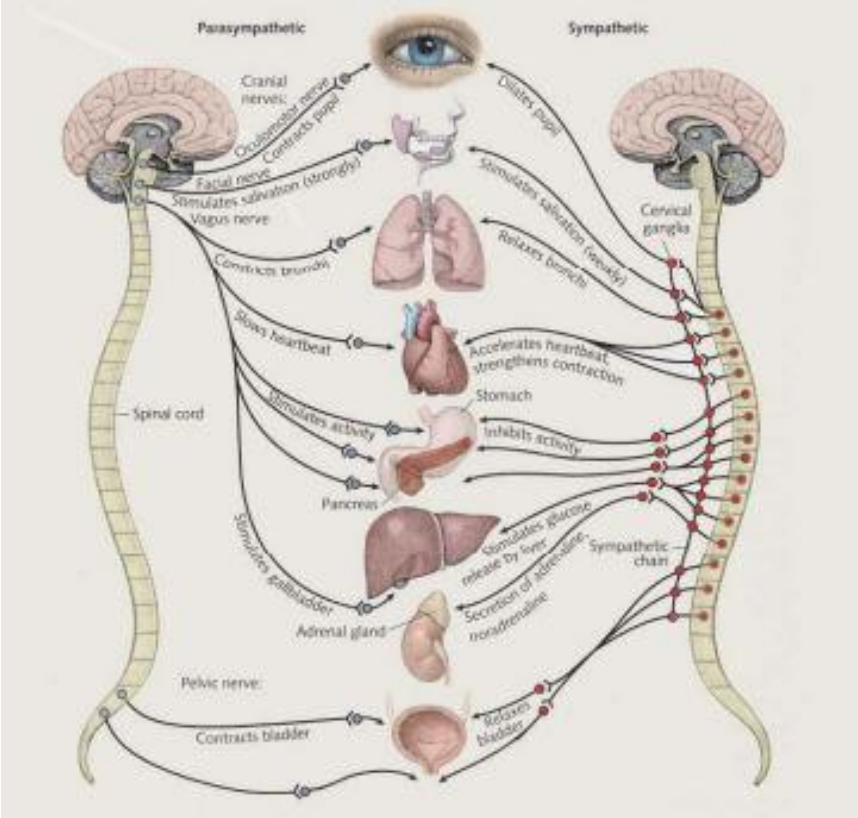

Fig. 1: The above figure shows how neural activity combines with hormones in the bloodstream to constitute the fight-or-flight response. (24)

2. The HPA Activation

The hypothalamic–pituitary–adrenal axis (HPA or HTPA axis), also known as the limbic–hypothalamic–pituitary–adrenal axis (LHPA axis) and, occasionally, as the hypothalamic–pituitary–adrenal–gonadotropic axis, is a complex set of direct influences and feedback interactions among three endocrine glands: the hypothalamus, the pituitary gland (a pea-shaped structure located below the hypothalamus), and the adrenal (also called "suprarenal") glands (small, conical organs on top of the kidneys). (25)

The interactions among these organs constitute the HPA axis, a major part of the neuroendocrine system that controls reactions to stress and regulates many body processes, including digestion, the immune system, mood and emotions, sexuality, and energy storage and expenditure. It is the common mechanism for interactions among glands, hormones, and parts of the midbrain that mediate the general adaptation syndrome (GAS). (26)

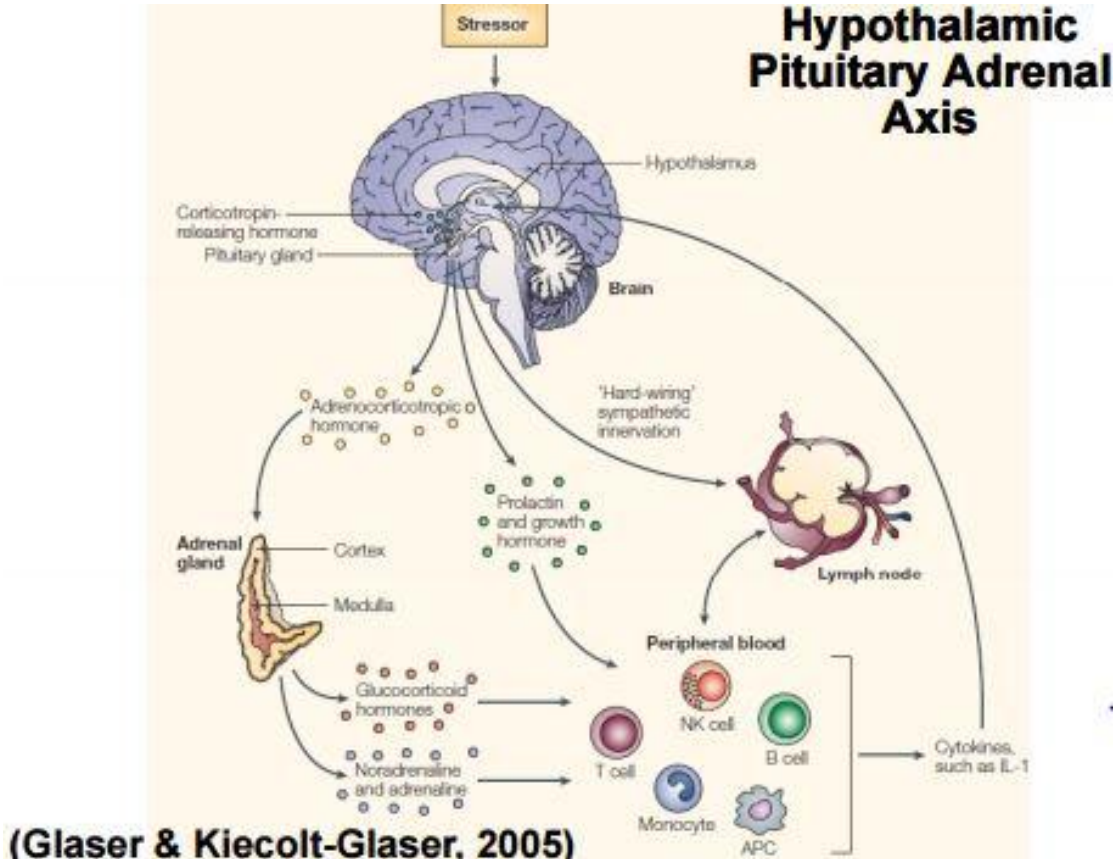

Fig. 2: The above figure shows the Hypothalamus-Pituitary-Adrenal Axis and its activation under stress. (27)

3. The Allostatic Load

The allostatic load is "the wear and tear on the body" which grows over time when the individual is exposed to repeated or chronic stress. (28) It represents the physiological consequences of chronic exposure to fluctuating or heightened neural or neuroendocrine response that results from repeated or chronic stress. The term was coined by McEwen and Stellar in 1993. (29)

It is used to explain how frequent activation of the body's stress response, essential for managing acute threats, can in fact damage the body in the long run. Allostatic load is generally measured through a composite index of indicators of cumulative strain on several organs and tissues, but especially on the cardiovascular system. (28, 30)

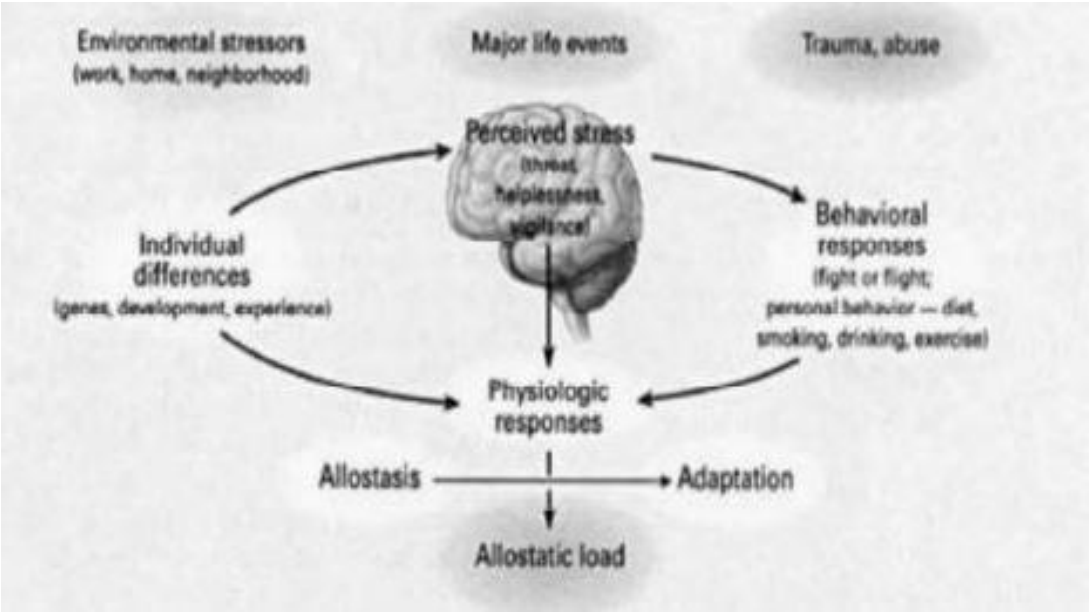

Fig. 3: The above figure represents the allostatic load. (31)

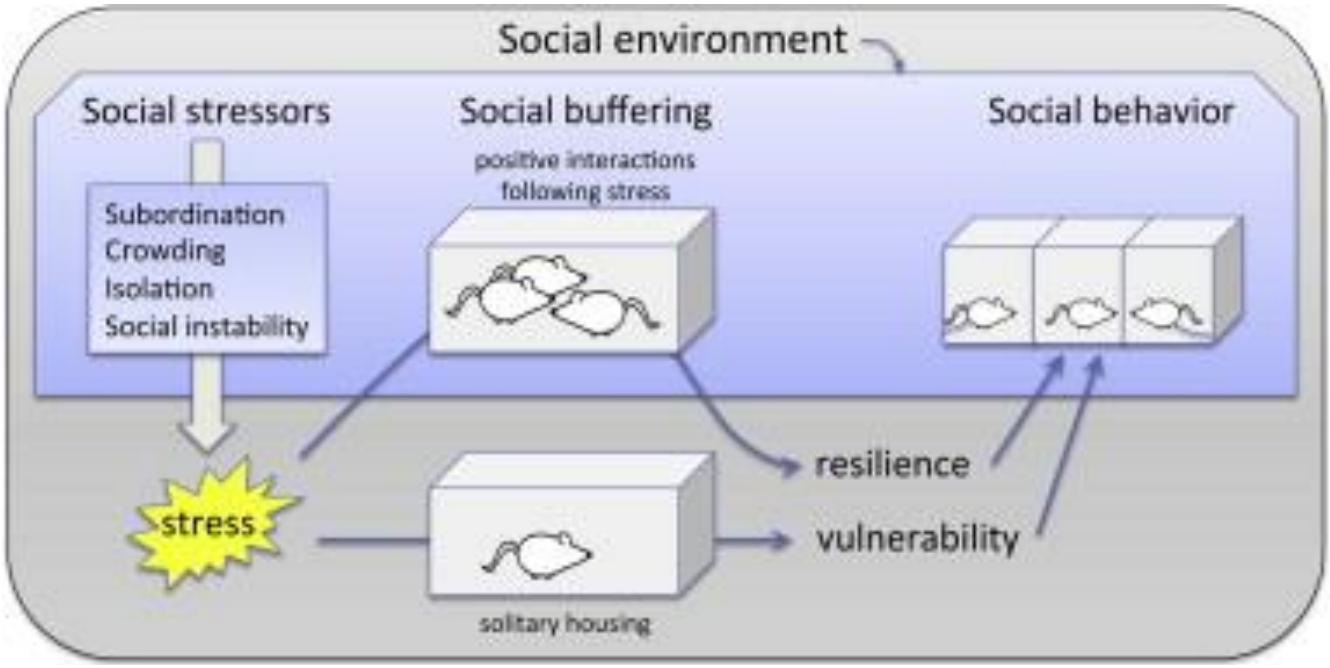

Fig. 4: Schematic representation of the levels at which the social environment impacts and reflects the individual. (18)

2.2) SOCIAL BEHAVIORAL TESTS

Most frequently used behavioral tests for mice are:

1. A simple social choice test in which the rodent is placed in the central compartment of the three interconnected chambers to assess social behaviour. The external chambers might include a tethered mouse on one side versus an empty chamber on the other side to assess interest in social interaction. (32)

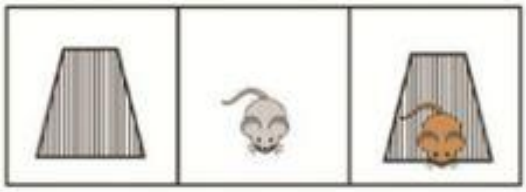

Fig. 5: Social recognition

2. A simple social choice test in which the rodent is placed in the central compartment of the three interconnected chambers to assess social behaviour. The external chambers might include a tethered familiar mouse on one side versus a tethered unfamiliar mouse on the other side to assess interest in social novelty. (32)

Fig. 6: Preference for social novelty

3. A test to assess the cognitive rigidity using a T-maze alternation task in which the tested rodent has to associate a particular response (for example going in the left arm) with the presence of a reward. Cognitive rigidity can be assessed by the ability of the animal to transfer this response when the reward is placed in the opposite arm of the maze. (32)

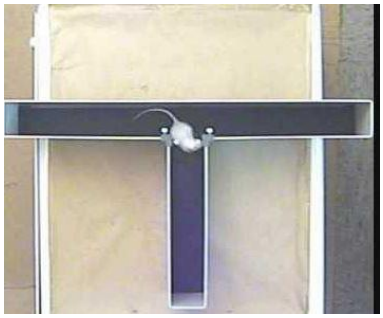

Fig. 7: Mice in a T-maze

4. The Morris Water Maze (MWM) is designed to test spatial memory and long term memory by observing and recording escape latency, thigmotaxis duration, distance moved, and velocity during the time spend in the MWM water tank. (32)

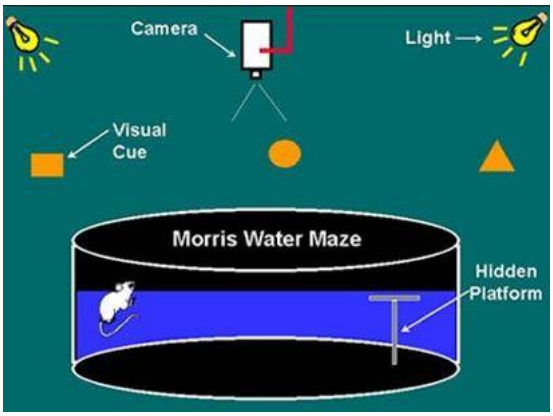

Fig. 8: Mice in a morris water maze

5. Another test is to assess anxiety behavior in the open arms of an elevated plus maze. Rats or mice are placed at the junction of the four arms of the maze, facing an open arm, and entries/duration in each arm is recorded by a video-tracking system and observer simultaneously for 5 min. An increase in open arm activity (duration and/or entries) reflects anti-anxiety behavior. (32)

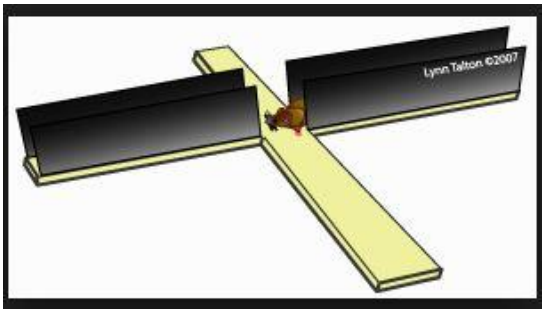

Fig. 9: An elevated plus maze

2.3) CHARACTERISTICS OF ANIMAL MODELS

There are three main characteristics of any animal model in order for it to be considered as a relevant model of a psychiatric condition described in humans. These are described as:

- Construct validity
- Face validity
- Predictive validity (22)

Construct validity mainly relies upon identity of causation between the animal phenotype and the human disease. It corresponds to the fact that both the behavior in the model and the features being modeled can be unambiguously interpreted and are homologous.

Face validity implies that the phenomenological aspect observed in the animal is similar to the one observed in patients. This might correspond to identical neuroanatomical, neurochemical, behavioural and cognitive modifications in the model compared to the human disease. It encompasses both treatment features and symptomatic aspects.

Finally, *predictive validity* means the ability of the model to accurately respond to the treatments that are employed. Predictive validity relies on whether a model correctly identifies antidepressant treatments of pharmacologically diverse types without making errors of omission or commission, and whether potency in the model correlates with clinical potency. (33)

2.4) SPECIFICATIONS OF ANIMAL MODELS

	RAT	**MOUSE**
Body weight (g)	200-300	25-30
Life span (years)	2-3	1-2
Water consumption (ml/day)	35	6
Food consumption (g/day)	20	5
Urine excretion (ml/day)	10-15	1-3
Oestrus cycle (days)	4-5	4-5
Gestation period (days)	21	19
Breeding age (days)	60-80	42-56
Breeding life (years)	1-1.25	0.75-1.5
Litter size	10-12	10-12
Body temperature (°C)	37.5	37.4
Respiratory rate	80-150	90-180
Heart rate (beats/min)	260-450	300-750
Blood pressure (mmHg)	130/90	120/75
Blood volume (ml/kg)	50-65	70-80

Table 1. Shows the specifications for rat and mice model (34)

2.5) GENERAL USES OF HYPERICUM AND FLUOXETINE

- ***Hypericum perforatum***

Since ancient times, the extracts of St. John's Wort (SJW) have been used for their medicinal properties. (35) SJW (also known as Hypericum) is *Hypericum perforatum*, a member of the Hypericaceae family. Traditionally, SJW has had a number of different uses including applying it externally as a treatment for wounds and burns, or taken internally as an infusion or herbal tea to treat fevers and nervous conditions including depression. (36) The therapeutically used *Hypericum perforatum* extracts contain numerous other bioactive components with diverse spectrums of pharmacological activity profiles.

- **Fluoxetine**

Fluoxetine is a selective serotonin reuptake inhibitors (SSRI) antidepressant. Fluoxetine affects chemicals in the brain that may become unbalanced and cause depression, panic, anxiety, or obsessive-compulsive symptoms. (37)

Fluoxetine is used to treat major depressive disorder,bulimia nervosa (an eating disorder) obsessive-compulsive disorder, panic disorder, and premenstrual dysphoric disorder (PMDD). (38)

CHAPTER 3

AIMS AND EXPERIMENTAL DESIGN OF STUDY

The aims of this study were to:

1) Create a chronic unpredictable mild stress (CUMS) that would resemble non-traumatic stress conditions in human beings;
2) Investigate the behavioral effects induced by CUMS in rats and mice;
3) Investigate the influence of psychoactive drugs-Fluoxetine and Hypericum on social behavior in mice and rats.

The study was divided into two parts: effect of acute stress and effect of chronic stress

- ❖ Effect of acute stress

The effect of acute stress was observed in normal rats that were not given any psychotropic drugs. Only the subject rats were made to suffer with acute stress by restraining them for 15 min. Their social interaction was then observed by means of the three chamber social interaction test.

- ❖ Effect of chronic stress

The effect of chronic stress was seen on both rats as well as mice.

The rats were divided into the following 4 groups:

- Control: No stress or psychotropic drug
- CUMS: chronic unpredictable mild stress
- CUMS + Fluoxetine:
- CUMS + Hypericum

The mice were divided into the following 6 groups:

- Control: No stress or psychotropic drug
- Control + Fluoxetine
- Control + Hypericum
- CUMS: chronic unpredictable mild stress
- CUMS + Fluoxetine:
- CUMS + Hypericum

Both the parts consisted of the same procedures before starting the CUMS protocol. Before starting the chronic unpredictable mild stress protocol, the body weights of both rats and mice were noted. Then they were subdivided into a stress and control group as mentioned above.

Mice and rats of the stress group were exposed to different socio-environmental stressors on a daily basis. The stressors followed an unpredictable order. The control group was left undisturbed.

The behavioral tests for the control group were performed earlier and that for the stressed group were performed after 21 days of exposing the group to stressors.

3 days after the CUMS protocol terminated, the rats and mice were capacitated and their organs, mainly liver, heart, kidney and brain, were preserved for future biochemical or histochemical testing.

CHAPTER 4

MATERIALS AND METHODS

In this behavioral study, where the mice were injected with specific drugs belonging to the class of antidepressants specifically Fluoxetine and *Hypericum perforatum*, we have made use of **Crawley's sociability and preference for social novelty protocol** to describe the influence of stress as well as the psychoactive drugs on social behavior in mice and rats.

Social interaction tests and their observation permit adequate measurement of a range of behavioral activities such as sociability, preference for social novelty and sexual preference. (39)

The social interaction test is a simple test in which behaviors are video-recorded and analyzed to assess active interaction time in a test mouse with a familiar and a novel mouse. In the three-chamber social approach task, social deficits are reflected by a lowered level of approach toward a cage that contains a novel mouse. The social interaction test requires manual scoring, and it may be more sensitive for detecting deficient social interaction in some cases. (40)

3.1) CHRONIC UNPREDICTABLE MILD STRESS PROTOCOL

The chronic unpredictable mild stress (CUMS) protocol has been widely used to study the impact of stress exposure in several animal models and consists in the random, intermittent, and unpredictable exposure to a variety of stressors during several weeks. CUMS paradigm has been long used to model depression, and consists in the continuous exposure of animals to stressful situations, usually for at least 4weeks. (41)

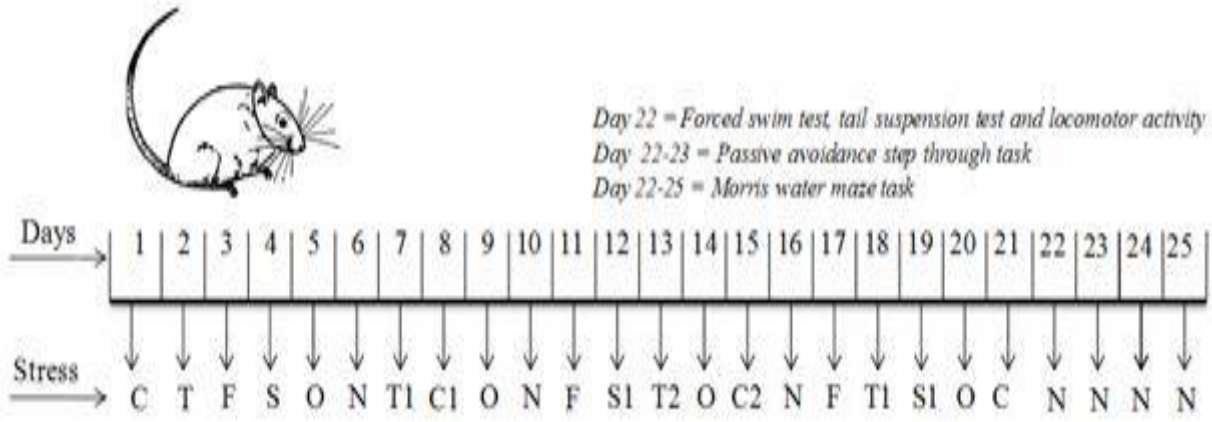

Fig. 10: The chronic unpredictable mild stress protocol. (32)

DAY	STRESSOR
1	Cold swim (8 °C, 5 min)
2	Tail pinch (1 min)
3	Food and water deprivation (24 h)
4	Swimming at room temperature (24±2 °C, 20 min)
5	Overnight illumination
6	No stress
7	Tail pinch (1.5 min)
8	Cold swim (10 °C, 5 min)
9	Overnight illumination
10	No stress
11	Food and water deprivation (24 h)
12	Swimming at room temperature (24±2 °C, 15 min)
13	Tail pinch (2 min)
14	Overnight illumination
15	Cold swim (6 °C, 5 min)
16	No stress
17	Food and water deprivation (24 h)
18	Tail pinch (1.5 min)
19	Swimming at room temperature (24±2 °C, 15 min)
20	Overnight illumination
21	Cold swim (8 °C, 5 min)

Table 2. The above table shows the stressors to which the animal models are exposed from day 1 to day 21. (32)

Starting from day 21 (60 min after drug administration), animals were subjected to different behavioral tests.

3.2) BEHAVIORAL ANALYSIS

❖ Materials

- *Subjects*: any strain of mice at least 3 months of age. Both male and female mice can be used. No prior training is required, though subjects should be acclimated to testing environment and experimenter before testing. Normal 5-day handling should be supplemented with 3 days of 10min/day habituation to pencil cup.
- *Object mice*: two per subject, no previous interaction with subject. Object mice may also require handling
- *Apparatus*: two 20 x 40.5 x 22cm three-chamber Plexiglass apparatuses with 10.2 x 5.4cm infrared-equipped portals between chambers.
- *Steel pencil cups (8)*: 10.4cm diameter, 11cm high, steel bars spaced 1cm apart. Two used as empty cups, six used to hold objects.
- *Plastic drinking cups (4)*: placed on top of steel pencil cups to prevent subject from climbing on top.
- *Video cameras on tripods (2)*: positioned one in front of each apparatus and angled so that video is taken from slightly above.
- *Privacy blind*: placed around apparatus to eliminate external room cues.
- *Proper lighting*
- *Water*: used to clean apparatus between trials.
- *20% ethanol*: used to clean apparatus and cups between subjects. (42)

❖ Detailed standing operating procedure (sop)

Before testing:

- *Acclimation*: subjects in home cage were placed in testing room for at least 1hr before testing to minimize effects of stress on behavior during testing.
- *Subject training*: none was required.

Testing procedures:

1. Habituation

- Doors to side chambers were closed and subject animal was placed in center chamber. Recording was started and doors were opened.

- After 10min of habituation, recording was stopped, subject was guided back to center chamber, and doors were closed. Urine was soaked up, fecal pellets were removed, and side chambers were cleaned with water. (42)

2. Sociability

- One steel pencil cup was added to each side chamber. Object mouse #1(male/female) was placed under cup in left chamber and the cup in right chamber was left empty. Plastic cups were placed on top of steel pencil cups to prevent subject from climbing on top. Recording was started and doors were opened.
- After 10 min Sociability trial, recording was stopped, subject was guided back to center chamber, and doors were closed. Cups were removed for cleaning and object mouse #1 was returned to home cage. Urine was soaked up, fecal pellets were removed, and side chambers were cleaned with water. (42)

3. Social Novelty

- New steel cups were added, one to each side chamber. Object mouse #1 (same sex as that of the subject mice) was placed under cup in right chamber and object mouse #2 (same sex as that of the subject mice) was placed under cup in left chamber. Same plastic cups were placed on top of steel cups. Recording was started and doors were opened.
- After 10 min Social Novelty trial, recording was stopped, subject was guided back to center chamber, and doors were closed. Subject was then returned to home cage, cups were removed for cleaning, and object mice #1 and #2 were returned to home cages. All cups and chambers were cleaned with 20% ethanol between subjects. (42)

4. Sexual preference

- New steel cups were added, one to each side chamber. Object mouse #1 (different sex as that of subject mice) was placed under cup in right chamber and object mouse #2 (same sex as that of the subject mice) was placed under cup in left chamber. Same plastic cups were placed on top of steel cups. Recording was started and doors were opened.
- After 10 min Social Novelty trial, recording was stopped, subject was guided back to center chamber, and doors were closed. Subject was then returned to home cage, cups

were removed for cleaning, and object mice #1 and #2 were returned to home cages. All cups and chambers were cleaned with 20% ethanol between subjects.

❖ **Data Analysis**

The following parameters were collected for analysis:

- Time in each chamber
- Entries into chambers
- Time spent sniffing each cup / Active-direct contact (40)

CHAPTER 5

RESULTS

❖ STUDY PART 1

The scoring sheet for social behavior in case of **acute stress** is as follows:
1) Control experiments from 19th to 31st dec-2014
2) Acute stress + Social behavior from 2nd & 3rd jan 2015

Date	Subject Animal No.	Sex	Weight (gm)	Novel object Chamber			Object animal Chamber (M/F)			Novel animal Chamber			Object animal Chamber (M/F)		
				No. of entries	Time spent (Sec)	Direct/active contact	No. of entries	Time spent	Direct/active contact	No. of entries	Time spent (Sec)	Direct/active contact	No. of entries	Time spent (Sec)	Direct/active contact
19-Dec-14	R1F	F	78	4	120	3	5	500	25	5	365	20	5	318	18
20-Dec-14	R2M	M	151	4	121	10	6	255	39	2	150	9	3	219	29
27-Dec-14	R3M	M	158	4	46	10	5	192	30	3	140	16	5	242	10
27-Dec-14	R3F	F	83	3	55	8	5	371	37	7	195	24	6	320	21
27-Dec-14	R3M	M	158				1	600	12	3	99	10	3	501	9
27-Dec-14	R3F	F	83	5	283	12	5	307	26	3	95	9	3	282	12
31-Dec-14	R1M	M	133	6	335	12	5	246	21	2	330	11	1	150	8
31-Dec-14	R2M	M	151	4	57	6	8	358	25	3	193	13	5	375	8
31-Dec-14	R3M	M	158	4	100	5	6	412	25	3	232	17	4	351	8
31-Dec-14	R1F	F	78	5	129	14	7	446	28	4	384	13	4	182	9
31-Dec-14	R2F	F	69	2	38	4	4	282	15	4	139	8	5	435	10
31-Dec-14	R3F	F	83	5	108	7	8	368	27	7	183	16	7	350	10
02-Jan-15	R1M	M	133	3	230	13	3	305	16	1	25	3	1	565	10
02-Jan-15	R2M	M	151	5	130	6	6 (M3)	243(M3)	17(M3)	5	211	10	4(M3)	305(M3)	10(M3)
02-Jan-15	R3M	M	158	2	30	3	2	515	16	4	520	11	4	60	7
02-Jan-15	R1M	M	133	2	426	7	1	190	6	2	550	13	1	10	1
02-Jan-15	R3M	M	158	5	80	7	7 (M2)	490 (M2)	17 (M2)	2	145	6	1 (M2)	410 (M2)	4 (M2)
02-Jan-15	R2M	M	151	3	415	10	3	100	8	1	455	8	1	75	6
03-Jan-15	R2F	F	69	5	112	8	5	370	16	6	258	17	3	230	4
03-Jan-15	R3F	F	83	5	60	7	7	510	22	5	390	21	5	188	12
03-Jan-15	R1F	F	78	4	130	9	3	398	11	3	145	6	2	430	4
03-Jan-15	R1F	F	78	1	170	3	2	400	6	2	285	6	2	265	7

Table 3: Scoring sheet for social behavior (acute stress)

Red color: Opposite sex (compared to that of subject animal)
Green color: Stress
Blue color: Unusual results

❖ STUDY PART 2 (a)

Scoring sheet for social behavior of rats in case of chronic stress is as follows:

CUMS time schedule: 11th Jan to 31st –Jan-2015

Groups	Caging
Control: M1, F1	Cage 1) M1, M2
CUMS: F2, F3	Cage 2) F2, F3
CUMS+ Flx: F4	Cage 4) F1, F4, F5
CUMS + Hyp: M2, F5	

Subject Animal	Novel object Chamber			Object animal Chamber (M/F)			Novel animal Chamber			Object animal Chamber (M/F)			S- O- N
	No. of entries	Time spent (Sec)	Direct/active contacts	No. of entries	Time spent	Direct/active contacts	No. of entries	Time spent (Sec)	Direct/active contacts	No. of entries	Time spent (Sec)	Direct/active contacts	
M1	3	108	13	2	406	26	3	100	13	3	481	11	M1, F1,M2
M1	4	68	10	5	280	24	3	196	18	3	176	10	M1, F2, M2
M1	5	65	12	7	279	26	4	158	18	3	222	11	M1, F3, F1
M1	5	123	12	4	256	24	2	280	15	1	121	8	M1, F4, F5
M2	5	230	6	4	148	12	1	105	6	2	292	4	M2, M1, F1
M2	3	69	11	4	312	25	2	90	15	3	241	12	M2, M1, F5
M2	4	97	6	6	399	21	4	255	6	2	46	4	M2, F2, F1
M2	4	195	19	4	152	21	3	77	13	4	166	15	M2, F3, F4
F1	6	92	10	6	273	17	3	259	15	4	110	9	F1, M2, M1
F1	3	89	12	6	230	25	4	144	10	4	190	8	F1, F3, F2
F1	1	23	4	5	391	28	5	170	19	5	187	13	F1, M2, F2
F1	3	24	4	4	385	25	3	260	28	4	128	16	F1, M1, F3
F1	4	93	15	5	325	15	3	103	18	2	69	9	F1, M1, F4
F2	3	53	8	5	353	31	1	365	8	--	--	--	F2, M2, M1
F2	3	28	3	2	23	4	5	73	11	6	354	13	F2, F3, F4
F2	3	105	10	5	302	24	3	250	13	1	67	2	F2, M2, F5
F2	3	120	5	2	123	8	4	297	19	2	139	8	F2, M1, F6
F2	--	--	--	3	426	13	3	52	6	5	465	11	F2, M1, F3
F3	3	221	13	2	200	21	4	248	19	5	109	14	F3, M2, M1
F3	3	35	6	6	346	36	5	219	20	5	123	17	F3, F2, F4
F3	3	86	9	4	326	14	3	303	22	2	132	18	F3, M2, F5
F3	6	91	11	9	414	36	6	184	18	4	183	17	F3, M1, F6
F3	4	35	9	9	307	38	5	89	16	4	299	24	F3, M1, F2
F4	3	30	6	5	368	33	3	155	16	2	257	18	F4, M2, M1
F4	5	144	13	6	345	29	3	137	11	5	371	12	F4, F2, F3
F4	2	62	7	6	292	23	2	103	11	4	231	10	F4, M2, F5
F4	2	83	11	2	348	14	4	184	11	2	180	8	F4, M1, F2
F4	5	185	21	6	260	30	3	218	17	4	324	13	F4, M1, F5
F5	3	45	5	4	351	16	2	65	8	2	324	7	F5, M2, M1
F5	--	--	--	4	489	28	4	79	14	5	278	16	F5, F6, F7
F5	2	6	2	6	227	33	2	32	6	4	370	13	F5, M2, F6
F5	4	109	14	4	366	19	3	306	8	2	103	3	F5, M1, F3
F5	4	42	12	5	360	18	2	47	9	3	322	11	F5, M1, F4

Table 4: Scoring sheet for rats under chronic stress

Red color: Opposite sex (compared to that of subject animal)
Green color: Represents social affiliation/sexual preference (Object-male; Novel-female and vice-versa)

❖ STUDY PART 2 (b)

Scoring sheet for social behavior of mice in case of chronic stress is as follows:

S.No.	Group
1-3	Control
4-5	Ctrl + Hyp
6	Ctrl + Flx
7-10	CUMS
11-14	CUMS + Hyp
15-18	CUMS + Flx

Subject Animal	Novel object Chamber			Object animal Chamber (M/F)			Novel animal Chamber			Object animal Chamber (M/F)			S- O- N
	No. of entries	Time spent (Sec)	contacts	No. of entries	Time spent	contacts	No. of entries	Time spent (Sec)	contacts	No. of entries	Time spent (Sec)	contacts	
1) M1	9	208	25	6	241	22	4	175	15	4	195	17	M1,M10, M19
							6	307	22	5	140	14	
2) M2	5	65	12	7	279	26	4	158	18	3	222	11	M2,M9,M12
							3	100	13	3	481	11	
3) M1	5	123	12	4	256	24	2	280	15	1	121	8	M1,M2,M4
							3	196	18	3	176	10	
4) M29	3	324 (308)	25 (24)	1	45 (43)	12 (11)	1	247 (260)	16 (17)	2	286 (301)	20 (21)	M29,F2,M27
							4	316 (333)	13 (14)	3	126 (133)	11 (12)	
5) M30	2	10 (11)	2	7	61 (64)	12 (13)	3	222 (234)	28 (29)	1	82 (86)	13 (14)	M30,F9,F7
							5 (6)	219(274)	21(26)	5 (6)	201(251)	15 (19)	
6) M31	5 (4)	365 (270)	55 (41)	1	267 (198)	27 (20)	3	445 (481)	29 (31)	1	48 (52)	4	M31,M32,M15
							3	131 (121)	6	2	78 (72)	7 (6)	
7) M4	6 (8)	178 (248)	22 (31)	7 (10)	139 (194)	17 (24)	7 (10)	276 (385)	26 (36)	8 (11)	111 (155)	16 (22)	M4,M8,M33
							9 (12)	127 (175)	18 (25)	7 (10)	75 (103)	9 (12)	
8) M5	3 (4)	144 (192)	14 (19)	6 (8)	248 (331)	26 (35)	7 (9)	245 (316)	24 (31)	3 (4)	141 (182)	13 (17)	M5,M21,M27
							6 (9)	178 (254)	10 (14)	3 (4)	183 (261)	10 (14)	
9) M6	8 (11)	126 (168)	23 (31)	9 (12)	205 (273)	32 (43)	8 (11)	189 (252)	38 (51)	7 (9)	130 (173)	21 (28)	M6,F3,F4
							5 (7)	260 (360)	16 (22)	5 (7)	84 (116)	15 (21)	
10) M7	9 (12)	126 (168)	17 (23)	11 (15)	255 (340)	33 (44)	7 (9)	196 (253)	18 (23)	7 (9)	208 (268)	13 (17)	M7,F4,M6
							5 (7)	238 (317)	8 (11)	2 (3)	26 (35)	5 (7)	
11) M18	4 (5)	65 (87)	10 (13)	3 (4)	143 (191)	15 (20)	3 (4)	245 (350)	15 (21)	2 (3)	132 (189)	10 (14)	M18,M20,M32
							2 (3)	212 (283)	10 (13)	__	__	__	
12) M19	2	170 (204)	18 (22)	2	302 (362)	24 (29)	2	242 (279)	14 (16)	3	213 (246)	9 (10)	M19,M31,M33
							2 (3)	347 (458)	9 (12)	1	20 (26)	2 (3)	
13) M20	5 (6)	273 (352)	16 (21)	3 (4)	159 (205)	11 (14)	4	265 (297)	10 (11)	3	201 (225)	9 (10)	M20,F7,M18
							1	366 (505)	8 (11)	__	__	__	
14) M20	5	416	35	3	110	13	6	361	21	6	236	14	M20,F6,F4
							3	465	19	1	22	11	
15) M21	2	425	31	-	-	-	6	207	17	3	108	12	M21,M23,M18
							2	48	5	2	185	12	
16) M22	3	302	28	-	-	-	1	80	6	2	345	20	M22,M33,M31
							2	65	6	5	250	21	
17) M23	2	108	10	1	230	13	3	85	8	4	224	11	M23,F3,M22
							5	61	5	3	99	9	
18) M24	3	160	17	4	180	20	5	155	31	4	101	14	M24,F7,F9
							5	197	13	4	155	9	

Table 6: Scoring sheet for mice under chronic stress.

In the following graphs, only a single parameter i.e. time spent in object/novel chamber, was taken into consideration as it gave the most reliable results.

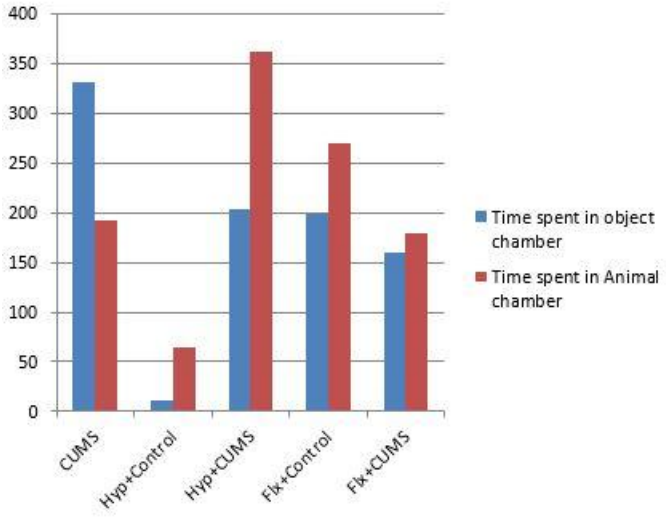

Fig. 11: Sociability

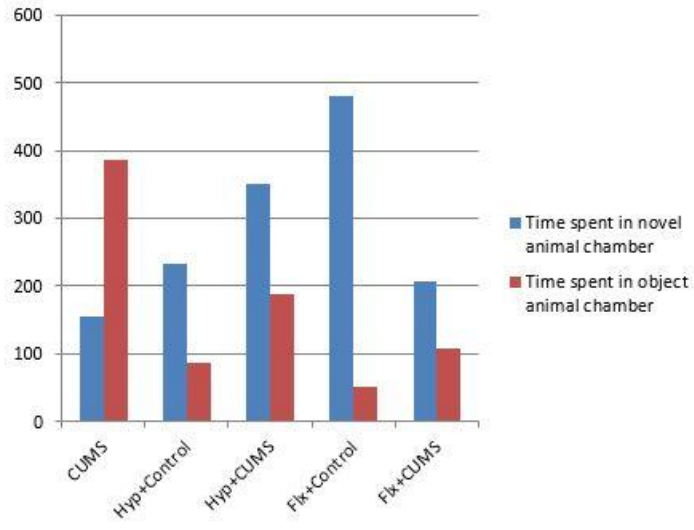

Fig. 12: Preference for Social Novelty

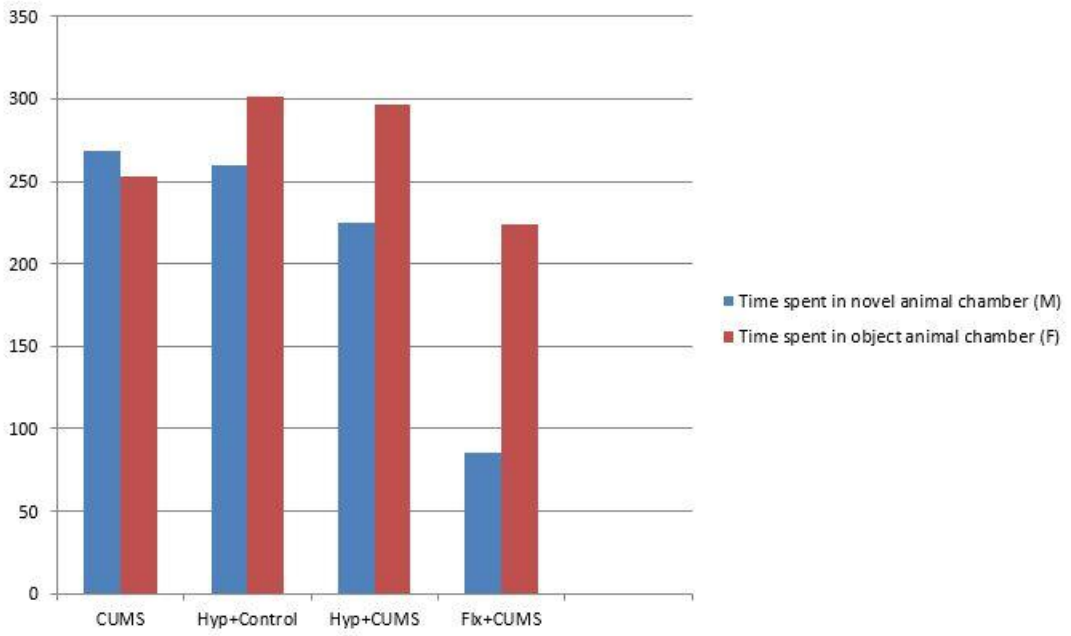

Fig. 13: Social Affiliation Analysis

CHAPTER 6

CONCLUSION & DISCUSSION

- **STUDY PART 1**

Conclusions:

1) Control non caring male (M1) prefers female over stressed male
2) Male cage-mate preferred stressed dyad over novel female.
3) Stressed male preferred male over female
4) Normal uncaring male (M1) avoids stressed male i.e. reduced empathy
5) Stressed male preferred familiar dyad over novel animal
6) Cage-mate male avoided non caring dyad (M1) in sociability and preferred novel animal later on
7) Control female preferred stressed female over novel male
8) Control female preferred novel male over stressed female
9) Stressed female has increased sociability and reduced novelty between same sexes.
10) Stressed female prefers female dyad over novel male.

- **STUDY PART 2 (a)**

Conclusions:

1) Sexual preferences in stress male changes drastically as compared to ctrl.
2) Stressed male prefers stressed female over ctrl female.
3) Stressed male prefers the familiar female i.e. reduction in social novelty.
4) Females prefer stressed male over normal male.
5) Social novelty inverts in stressed females.
6) Females prefer stressed females over novel normal male.
7) Females prefer normal males over cage-mate dyad

- **STUDY PART 2(b)**

Conclusions:

1) Evident shifts in social behavior were observed upon exposure to chronic stress.
2) Normal social behavior was observed upon treatment with *Hypericum perforatum* and Fluoxetine.

CHAPTER 7
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