The philosophy of a science determines the methods it uses. The comparison of nature/type of methods used in traditional physiological psychology, psychobiology, health psychology and psychoneuroimmunology reflects their philosophies. Advances in the neurosciences over the past four decades revealed that the immune and endocrine systems are profoundly affected by the emotional state and, conversely that these systems can change the emotional state, has helped to refocus the attention of researcher on the interrelationship between the mind and the body Robert and Nicholas C. (1995). The way mind and body are assumed to interact governs the structure of research. Do we look for electrophysiological evidence of mental events? Do we conceive of these two concepts, mind and body, as different kinds of worlds, following somewhat different kinds of laws? or are the two just different labels for same thing?

Over the past fifteen years the functional anatomy of immune system has been successfully challenged. Certainly, immune system has idiosyncratic, self regulatory properties and functions as does the nervous system and endocrine system. Each has evolved to respond to specific stimuli originating from internal to
external environment. In this sense, it has been linked to an additional sensory organ. Yet, researches Ader, Felter and Cohen (1991) suggest that immunoregulatory processes are part of an integrated system of defense. The integration is reflected in the development of psychoneuroimmunology study of behavioural-neural-endocrine- immune system interaction. There exist reciprocal relations between neural and immune functions, endocrine and immune function and behaviour and immune functions as shown in Figure 1 below:

**FIGURE 1**

*Relationship among brain, behaviour, nervous, immune and endocrine systems.*

Since psychoneuroimmunology involves the interaction between the central nervous system (CNS), endocrine system and immune system. Thus, the research methods to be employed depend on the emphasis of research.

With the purpose of clear-cut categorization and understanding, the present chapter has been presented in different sections dealing with - methods for studying CNS (brain) functioning, *measures of immune functions*, hormones and different techniques used for behavioural measurement.
Methods for Studying CNS Functions:

This section describes not only the most commonly used physiological and behavioural methods but also the most advanced techniques to investigate central nervous system functions.

The most commonly used physiological methods involve lesioning a part of brain and measuring the resulting changes in behaviour; electrically or chemically stimulating a part of the brain to see what kinds of behaviour are produced and recording from various parts of the brain when the organism is displaying various types of the behaviours. Other kinds of physiological techniques have been limited to one or two areas of brain research may be discussed. Besides these, diverse kinds of psychopharmacological procedures and chemical assays of brain tissue would be taken up for discussion. Another brain techniques used in a lesion techniques when a destruction or fundamental disruption of the brain and when a lesion leads to a deficit in some behaviour, it is assumed that a damaged area had an important role in the control of that behaviour. One of the greatest difficulties in lesion method is to describe the behavioural deficit after a lesion. It becomes very important to examine the location of lesion in the intended location or somewhere else. This can be accomplished by applying a histological stain to the brain that colours the cell bodies or axons.

In stimulation studies, parts of the brain are stimulated electrically chemically or inhibited by injecting chemicals while ongoing behaviour is simultaneously observed. Stimulation techniques may be of macrostimulation or microstimulation type. In macrostimulation many nerve cells within a given area of the brain are simultaneously activated or inhibited by electrical or chemical stimulation. Whereas, in microstimulation, one or at most a very few cells are electrically or chemically stimulated or inhibited. When one very tiny microelectrode or microcannula is inserted inside and another outside the same cell, intracellular
stimulation is being done. One of the problem involved with
stimulation technique is to limit macrostimulation to a ‘brain
part’, since chemicals do diffuse away from the site of their
injection. Thus the possibility always exists that the stimulation
effects come from activating parts of the brain other than the
‘targeted’ area. Another problem relates to microstimulation during
which large nerve cells are much more likely to be stimulated
than are the small cells.

The Electrophysiological methods record the electrical activity
of brain nerve cells while simultaneously observing behaviour or
while presenting various kinds of stimuli to experimental organism.
Recently, electrophysiological studies (Guyton and Hall, 2003)
have been focusing on induced changes in ion-currents, fluxes of
calcium, potassium or sodium, membrane potentials and neuron
firing. Electroencephalography (EEG) has been involved in some
studies (Windhorste and Johansson, 1999). It enables the
investigators to make gross determinations of brain activity in
humans and other animals without actually cutting into the skull.
The gross electrode, generally eight or fewer are attached with
glue or other adhesive to various locations on the surface of
scalp. Electrodes may be placed directly on the brain itself. The
output of those electrodes is then amplified and recorded. Most
of the electrophysiological methods can be applied to both neurons
and white blood cells (leucocytes) Robert and Nicholas (1995).
Hence, the action of neurotransmitters and hormones on
electrophysiological characteristics of leucocytes and the action
of cytokines (the complex proteins which regulate cellular
interactions and functions and contribute to cell growth and
differentiation) on the electrophysiological property of
hypothalmic and hippocampal in CNS neurons, can be determined.

Another type of the gross recording, either from the surface
of the brain or from a lower part of the brain, involves an evoked
potential. This is the change in the grossly recorded electrical
activity of one part of the brain, a change that is evoked by the presentation of some stimulus or by the occurrence of some response. Microelectrode recording can reveal the details of the pattern of electrical activity and to identify which nerve cells are displaying that activity by injection through electrodes. But interpreting records made from gross electrodes entails several problems. First EEG are largely limited to what large nerve cells are doing, no information about the activity of smaller nerve cells. Second, the nerve cells closest to electrodes will contribute more than those which are nerve cells farther away. Third, the gross recordings cannot distinguish a pattern involving stimulation of neurons progressively closer to an electrode from a pattern involving inhibition of neurons progressively farther from the electrode. Technological breakthrough in neuroimaging - such as Positron Emission Tomography (PET) functional magnetic resonance (FMRI). Single Photon Emission Computerized Tomography (SPECT) gives us the opportunity to pinpoint areas of the brain that are responsible for generation of emotions, thoughts and even higher abilities of the mind e.g. meditation and prayer. (Moye, Richardson, Postwhile & Justice, 1995, Katrina, 2006)

Intracellular recording and extracellular micropressure ejection are the techniques which are suitable for recording the excitability of neuronal membranes. The patch clamp technique is useful to determine potassium (K+), Sodium (Na+) or Calcium (Ca2+) current in the whole cell configuration following incubation with cytokines. The Ca2+ current is directly related to neuronal excitability in hippocampus. Its suppression results in the stress effects of interleukins, that is observed following a bacterial viral infection.

Cytokines are the complex types of the proteins which are produced and secreted by a variety of cell types throughout the body including the brain where microglia and astrocytes or part
of the immune system. Cytokines that control function of immune system are interleukins. The cytokines also activate the hypothalmic pituitary-adrenal axis. This suggests that changes in endocrine function that occur during immune activation may be partly modulated by interleukins. So far, the location of cytokine centres and cytokine binding sites in the brain is unknown. But the location of interleukins has been studied by using the methods like immunoautoradiography, radioimmunoassay and immunofluorescence micrography. To characterize the distribution of cytokine like immunoreactivity, antibodies which recognize rat cytokine immunoreactivity (IR) and the Tac antigen-IR of the human cytokine receptors are administered. On the brain slices immunoautoradiography is performed. These brain slices are coronal sectioned and preincubated in Tris-NaCl buffer. The brain slices are then incubated with antiserum directed against recombinant cytokines. This antiserum has been shown to detect rat cytokine-immunoreactive material in a radioimmunoassay of tissue from different brain regions. If the cytokine antiserum is preabsorbed with recombinant cytokine, cytokine immunolabelling of brain sections would be decreased.

To localize the cytokine receptors, the brain section is incubated in sheep serum and then with a monoclonal antibody directed against Tac antigen of the cytokine receptor. If the antibody is preabsorbed with recombinant cytokine immunolabelling of brain sections with anti Tac monoclonal antibody is undertaken. For cytokine receptor autoradiography, the brain is removed and stored at -80°C, coronal sections are then cut into gelatin-coated slides. These sections are incubated in buffer containing radiolabelled cytokine. By using a computerized microdensitometer the quantification of cytokine immunoreactivity and receptor localization are made. It has been observed that interleukin-1 (IL-1) receptors are localized in neurons in the hippocampus, cortex, cerebellum and in the anterior pituitary. These are the areas which are involved in IL-1
stimulatory effect on ACTH secretion. The highest densities of interleukin-2 (IL-2) have been evident to the median eminence, hippocampus and cerebellum. These are the locations which may be related to affective modulation and memory functions. Cytokine mRNA expression and gene expression have been associated with the inflammatory status of brain. The physical, genetic and infection-induced changes in brain cytokine expression may have a causal relationship to psychiatric diseases (Neumann, 2005). For studying the cytokine mRNA expression in the brain, an in situ hybridization histochemical technique has generally been used. Another technique that is popular to study expression of pro-oncogene of mRNA is immunocytochemical staining.

For quantification of biogenic amines, their metabolites and precursors in different brain regions, brain slices and specific brain nuclei, the high performance liquid chromatography with electrochemical detection is normally employed. This system is even able to assess the concentration of noradrenaline, adrenaline in the noradrenergic system and of dopamine, homovanillic acid and others in dopaminergic system. In serotonergic system, 5-hydroxytryptamine, tryptophan and others can be quantified. The limitation of this system is that the results only reflect neurotransmitter changes in whole tissue or specific brain regions.

Peptidomies is a methodological approach to study the endogenous peptides. It is a new method using a combination of high resolution mass spectrometry and separation techniques such as capillary electrophoresis and nano liquid chromatography. The micropunch technique is better than this system, in which brain is cut into several slices with a freezing microtome, and then according to a brain map, different sizes of needles are used to punch out the specific nuclei position. Another technique microdialysis probe is specifically suitable to the studies related to free moving animals (Katrina, 2006). The microdialysis probe or push-pull cannula is inserted into specific nucleus in which the neurotransmitter changes to be studied for example following
sheep red blood cell, cytokine or lipopolysaccharide challenges. The perfusion of artificial cerebrospinal fluid is made through the push tube after the recovery of the animals from surgery. It enables the perfusate or dialysate samples to be assayed by high performance liquid chromatography with electrochemical detection.

**Measures of Immune Functions**

The immune system is a complex set of tissues with mobile elements, whose function is to protect the organism by exogenous microscopic life forms or particles and to rid the body of defective, damaged or malignantly transformed cells. It is composed of variety of cell types which can be classified according to size, shape, appearance, staining properties and functional properties. The type of proteins present on the cell’s membrane or within its cytoplasm also differentiates the cells into types (Brannon & Feist, 2000).

The cell types of the immune system derive from hematopoietic stem cells located in the bone marrow which can be designated megakaryocytes and leukocytes. The subtypes of these classes are the granulocytes which include neutrophils (colourless), eosinophils (red) and basophils (blue) based on the staining characteristics. The immune response repertoire includes the complement system consisting of twenty proteins that are produced primarily in the liver and released into the circulation. The natural killer cells are the large granular lymphocytes capable of cytotoxicity. These cells participate in the process of eliminating the infected or defective cells. The antibodies are produced by β lymphocytes, which undergo much of their maturation within the bone marrow and complete it in the lymph nodes within the microenvironment of the lymphoid follicle. T lymphocytes helper T lymphocytes (CD4+) are present. The immune system is composed of thymus, spleen, lymphnodes, lymphatic vessels,
tonsils, adrenoids and most important the bone marrow which manufactures all the cells that eventually develop into T cells, B cells, phagocytes, macrophages and natural killer (NK) cells etc. (Song & Leonard, 2000).

Immune activity is a multifaceted process occurring at a multitude of sites throughout the body. In many ways, the immune system is as complex as the central nervous system and, thus, defies simple characterization in terms of its function. Numbers of measures have gained popularity in the research to gain a foothold on the relation between immune function and various challenging conditions affecting the individual. This section discusses the more frequently employed measures.

In vivo tests involve activating the immune system by introducing a novel antigen (as in immunization) and then, after an appropriate period has elapsed, assaying for antibodies to the specific antigen in the serum of the individual. The most commonly employed assay is known as enzyme-linked immunosorbent assay (ELISA). Serum from the individual is incubated on a surface with the specific antigen bound to it. Antibody in serum specific for the antigen, if present, will bind to the surface. Another antibody is then introduced that binds to human antibody molecules and carries an enzyme, which catalyzes the reaction of a colourless substrate to a coloured product. The intensity of the colour that appears is quantitatively related to the amount of specific antibody found to the antigen. The ELISA has a variety of other applications, which include detecting antigen (as opposed to antibody) in serum or quantifying cytokine released by activated lymphocytes. Antibody to tissue-bound antigens can be quantified using an immunofluorescence assay. This is similar to the ELISA procedure except that the starting point is actual tissue samples, exposed to serum and then incubated with antibody to human immune globulin that fluoresces in response to ultraviolet light.
In vivo assay examines the response to antigens that can be expected to have infected most individuals before testing. This method is known as the delayed hypersensitivity skin test. The antigens are introduced by needle puncture of the skin. Their processing is accomplished by macrophages and dendritic cells and presented to CD4+ memory cells that remain from previous encounters with the antigens. The CD4+ cell releases cytokines and attract neutrophils into the tissue, causing an elevated red area (inflammation) on the skin within approximate time of forty eight hours. This is the method used to evaluate for previous infection with Mycobacterium tuberculosis and in that particular, case is known as the tuberculin test (Moye, Richardson, Postwhile & Justice 1995).

The quantification of major classes of immunoglobulins like IgG, IgA, IgM, and IgE is being done by a number of in vitro quantitative assays. Nephelometry a technique that uses antibodies against the heavy chain of major classes of antibody molecules to form immune complexes that precipitates and yield estimates of the concentration of a particular class of immunoglobulin - IgG or IgA in the serum of an individual. The measurement of antibody specific for a given pathogen requires the ELISA technique that has been previously described.

The assessment of leukocyte subpopulations as well as lymphocyte subtypes, banks upon the techniques such as flow cytometry (Katrina, 2006). The cells are prepared so that they can be fed one at a time through a device with a laser that permits differential detection based on size and granularity of cells. Further, they can sense tags (colour-emitting dyes on antibodies specific for key surface molecules) such as CD3 or CD4 on the cells. Essentially, this device can separate and count cell types at a rate of 10,000 per second.

The in vitro functional tests can be used to quantify the ability of lymphocytes to proliferate. The cells are incubated with tritiated
thymidine, which is incorporated into the DNA of dividing cells, and are stimulated to divide using mitogens which are the nonspecific substances that induce mitosis, such as phytohemagglutinin, concanavalin A, and pokeweed mitogen. The amount of radioactivity detected provides an indication of the amount of cell division (proliferation) in the culture. The NK-cell activity can also be calibrated by incubating the NK cells with target cells that have been labeled intracellularly with radioactive chromium. The ability of NK cells to lyse the target cells causes a release of radioactive chromium and provides a measure of NK-cell cytotoxicity.

The above mentioned approaches to estimate the functioning of immune system or responsiveness are limited due to the reason, in humans, the variation that has been observed is well within the normal range and possibly not clinically significant. Moreover, except for skin testing, the measurements are based on peripheral blood samples, which may not adequately reflect what is transpiring within the lymphoid tissues or at sites of infection or malignant transformation.

**Methods for Quantification of Hormones**

It is clear at this stage that the immune defenses and that immune responses requires intercommunication by a variety of cell types. The molecular signals capable of enhancing and suppressing immune responsiveness not only originate within the immune system but can also arise from other systems. The available evidences Hanssen, Hasselquist, Folstad & Erikstad (2004) suggest that multiple signals must converge for triggering, sustaining and terminating an effective immune response. The release of endocrine glands is to a significant extent under neural control, is subject to negative feedback and is influenced by other hormones. To supplement, cytokines in immune cells alter the endocrine activity are now known to be released also by pituitary
cells, endothelial cells, glial cells and even neurons. Moreover, leukocytes have shown to produce pituitary hormones and other peptides to influence neuroendocrine activity. Although most pituitary hormones can be secreted by various tissues including the thymus, the disruption of pituitary supply has a major effect on immune function. To put it differently, the local effects of such hormones can rapidly fade if an organism is not able to sustain a systemic level, which in effect may act as a signal of viability of the organism.

Hormonal influences appear as important background signals capable of sustaining and modulating the massive metabolic undertaking that constitutes a functional immune system. This sort of role seems most in line with the effects of insulin, growth hormones (GH), prolactin (PRL), thyroid and parathyroid hormone (PTH). Most of the hormone changes have been measured in the plasma or serum, but such changes can also be determined in the supernatant of tissue homogenates, such as the brain and immune organs. Leucocyte-produced hormones are normally assessed in cell culture medium or in whole blood after culture. ACTH, cortisol and corticosterone assay the blood is mixed with EDTA or another anticoagulant, centrifuged and the plasma quickly frozen and stored at -70°C. Plasma ACTH, cortisol or corticosterone are subsequently determined in duplicate using commercially available assay kits. The Plasma corticosterone concentration may also be assayed by a fluorescence method. In this method, samples are mixed in chloroform. The chloroform phase is then transferred into a tube containing sulphuric acid: ethanol (32.5:17.5) and mixed. The samples were then kept in the dark for 45 min. An aliquot from the lower acid phase was removed and fluorescence measured in a spectrophotofluorimeter. (Daruna, 2004)

The Growth hormone (GH) may be assayed by radioimmunoassay. Purified GH is labelled with 125I. The NIADDK reference preparation rGH-RP-2 is used as a standard,
and the antiserum used is NIADDK-anti-rGH-S-5. Addition of label is delayed by 2 hours; this non-equilibrium protocol increases the sensitivity of the assay. Separation of the antibody-bound from free hormone is achieved by addition of protein A.

**Prolactin (PRL) another hormone concentration in blood:** Concentration is determined by a radioimmunoassay using anti-rat antiserum and rat PRL standard. The PRL assay sensitivity is 0.4-1.1 ng/ml. Radioimmunoassay kits are also available for human samples.

**Behavioural Measurement in Psychoneuroimmunological Research:** The experience of weakness, malaise, inability to concentrate or hypersomnia, anorexia, depressed activity and loss of interest in daily activities are the non-specific changes which may be labeled as ‘sickness behaviour’. Experimental evidences (Song Leonard, 2006) attribute these behavioural changes to the direct mediation of the CNS by the immune system as a consequence of infection. Cytokines are used to treat various malignancies, infectious diseases, neurodegenerative diseases and cancers. It has been noted that during treatment many adverse neurological and behavioural changes appear. It can be inferred that cytokines mediate behaviour is by changing central neurotransmission and/or by modulating endocrine functions. Although it is not so clear that behaviour can change immune function but there is evidence exhibiting immunomodulation through behavioural conditioning. So the changes in immune function affect behaviour. Immunological changes may result in hyperactivity, sedative behaviour, anxious behaviour, stressed response, and even memory impairment.

The variations in the immune system may lead to many symptoms which resemble different psychiatric states in both humans and animals. These models are established by systemic or central administration of cytokines, bacteria or virus. Moreover, the effect of ageing on the activity of the thymus and autoimmune
diseases may have a causal relationship with immune disorders evident in psychiatric diseases. Most of the models for investigation of stress or psychiatric diseases can be employed in psychoneuroimmunological research. The novel experience, electric footshock, noise, illumination versus darkness, restrain or predator exposure, temperature, the effect of physical stress, stressful life events, and other forms of the stress are frequently use approaches.

Depression models include developmental models, learned helplessness, respirine induced depression. Many stressors lead to anxiety and may be used to develop anxiety models. Schizophrenia models can be developed by using stimulant amphetamines and psychotomimetics like phencyclidine. Alzheimer’s disease can be initiated by changing central cholinergic function or by neurotoxin lesions.

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