CHAPTER IV

SUMMARY

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Corticosteroids (glucocorticoids and mineralocorticoid) are produced in the adrenal gland and its physiological significance began to be appreciated as early as 1855 as a consequence of the destructive disease of the adrenal glands. Corticosteroids became a subject of interest primarily because of two reasons (a) involvement in certain pathological conditions namely Addison's disease and Cushing's syndrome and (b) it's then suggested role of protecting the organism against stressors. The interest in this field continued from then on till now with in between ups and downs. Today however, it is perhaps one of the most widely researched topic, for its complexity in the mechanism of action in the field of molecular endocrinology and biochemistry.

It is now fairly established that the elevated levels of glucocorticoid (cortisol/corticosterone/cortisone) alters the cell metabolism to increase the resistance to stress by enhancing defensive/protective mechanisms to prevent further damage to the cells or tissue. On the other hand basal levels seem to play an important role in regulating the growth of the tissue. During development, it is involved in chemical differentiation of specific cell types, absolutely required for the survival of some neurons. It also play an important role in the induction of enzymes such as tyrosine hydroxylase, typtophan hydroxylase and glycerol phosphate dehydrogenase *etc.* in the brain as well as other tissues.

The effects of glucocorticoids (corticosterone in case of rats and

cortisol in case of human) are observable in as short time as in seconds to minutes and as long as hours to years. These effects have been attributed to its effects at non genomic and genomic levels.

The effects mediated through non genomic mechanisms involve direct intercalation of glucocorticoid in the membranes and/ or through membrane receptors, a recently postulated recent concept. The genomic effects of glucocorticoid are through cytosolic receptors. In brain they are of two types- type I which has high affinity for corticosterone and are generally saturated with basal levels of circulating corticosterone. On the other hand type II receptors have 3- 10 fold less affinity for corticosterone and are activated when levels of glucocorticoid are elevated . The total number of receptors and types of receptor distribution vary in different brain regions. Hippocampus has the highest number of total as well as type I receptors. Cerebellum has not only low number of total receptors but has extremely low levels of type I receptors (McEwen, 1982). The striatum although has fair number of total receptors, it has highest amount of nuclear retention of glucocorticoid compared to several regions studied.

The binding of the glucocorticoid to its receptor activates the receptor. This active hormone- receptor complex, which is a specific DNA binding protein and acts as transcription factors, which can enhance, activate or suppress.

Several observations on brain function indicate that glucocorticoid alter neuronal cell excitability. Elevated levels decrease and depleted levels increase the excitability. Apart from this, it decreases glucose transport in neurons and glia in a specific region, alters neurotransmitter

metabolism. The effects involve changes in enzyme activities such as tyrosine and typtophan hydroxylases, release and uptake of neurotransmitters. Decrease uptake of GABA with elevated glucocorticoid and neurotransmiiters such as NE/histamine stimulate cAMP which are secondary messenger. Elevated levels of glucocorticoid diminish the increase in cAMP levels with stimulation and cAMP levels are exaggerated in depleted levels of glucocorticoid. These effects are region specific.

Several studies have been carried out in this line to understand how glucocorticoid influence the levels of cAMP. The studies reported in literature reveal that no direct parameters such as receptor number (β - adrenergic) nor the adenylate cyclase or phosphodiesterase or the subunits of G-protein namely G_s or G_i change. However, very recent studies show a possible alteration in association of α,β,τ components and ADP ribosylation factors are considered responsible. Most studies imply a possible change in the membrane constituents which perhaps may potentiate or hamper the cascade phenomenon without actually affecting the levels of the above parameters.

The possible factors could either be a change in protein and /or phospholipid concentration, turnover or their changes in fatty acid composition or changes in the protein with respect to their phosphorylation - dephosphorylation states.

It was felt that the phospholipids could possibly be altered as several studies on non- neuronal systems suggest such a possibility. Glucocorticoids alter HMGCoA reductase activity in HeLa cells, changes

desaturase enzyme in toad bladder and liver, increases sphingomyelin synthesis in fibroblast and fat ghost,inhibits phosphoinositidase C in basophilic cells *etc.*

It was therefore felt worth investigating the effects on lipids. The studies were aimed to investigate with following questions in mind.

(a) Do circulating glucocorticoid play any role in maintaing brain phospholipids or their turnover and is it age related?.

(b) Do these effects of glucocorticoid relate to the type and distribution of their receptors in different brain regions?.

(c) Do permissive (basal) and excess of glucocorticoid have opposite effects on phospholipid levels or its turnover or fatty acid composition?.

(d) If there are any changes in phospholipids, are these associated with any changes in the membrane fluidity?

(e) Do the activities of membrane bound enzymes such as Na+K+ATPase, 5'Nucleotidase and acetylcholine esterase get affected with changes in the circulating levels of corticosterone?.

(f) Do the physiological alteration in circulating glucocorticoids (for example- ageing), also influence membrane bound enzymes. If so, can these changes be modulated by altering the circulating levels of glucocorticoids?.

(g) What are the histological observations of hippocampus in young and aged adrenalectomized rats?.

The experiments are designed to study the effects of elevated levels by giving S.C. 40 mg/Kg body wt. injection of corticosterone at different ages 10, 20 and 40 day postnatal days and adrenalectomized at different

ages- 20 day, 40 day, 2 month and 17 month old animals for a varying period and replacement study was carried out by giving 10 mg/kg body.wt, to adrenalectomized rats.

The regions studied include - whole brain, olfactory bulb, cerebellum, brain stem, hippocampus, striatum and cortex.

The parameters studied include estimations of subclass of phospholipid namely phosphatidylinositol- 4,5-bisphosphate, phosphatidylinositol -4- phosphate,phosphatidyl inositol,phosphatidyl serine,sphingomyelin, phosphatidyl choline,phosphatidyl ethanolamine and ^{32}P incorporation into the same, estimation of cholesterol, and galactolipids, fatty acid composition and membrane fluidity in the synaptosomal membranes. Membrane bound enzymes namely Na+K+ATPase, 5' nucleotidase and acetylcholine esterase in crude membrane preparation from above mentioned regions under different experimental conditions as well as synaptosomal and myelin fraction from cerebral cortex and cerebellum. Histological studies of hippocampus from young and aged adrenalectomzied rats.

The results obtained are summarized as follows.

(1) By monitoring parameters which reflect changes in circulating levels of corticosterone namely liver weight, thymus weight and plasma Na+ levels, it was assured that the paradigm used in the present study indeed alters the circulating levels of corticosterone.

(2) The initial experiments on whole brain aimed to investigate phosphoinositides metabolism reveal that as far as fractions of phosphoinositides are concerned at all ages as well as with adrenalectomy, the con-

centrations remain unaffected but turnover as judged by ^{32}P incorporation clearly indicate changes in the rates of incorporation. It increased with corticosterone treatment and decreased in depleted conditions. These inverse effects indicate a possibility that corticosterone levels regulate the phosphatidyl inositol metabolism. The effects are either on PI = PIP = PIP_2 exchange and/or phospholipase C mediated effects. In this connection, phosphatidyl inositol specific phospholipase C is known to be inhibited in dexamethasone treated basophilic cells (Her *et al.*, 1991). Duman *et al.* (1986) has reported that basal levels of inositol phosphates change with chronic adrenocorticotropin hormone treatment. It is also likely that phosphomonoesterase as well as phosphtatidyl inostiol kinase which are membrane bound are also under the influence of corticosterone.

With regard to other subclasses of phospholipids, at younger, i.e., 10 and 20 day postnatally elevated levels of corticosterone affect their concentration whereas at older ages, i.e., day 40 or adrenalectomized animals no such effects are seen. The phospholipid subclasses such as phosphatidyl choline and phosphatidyl ethanolamine increase at day 10 whereas phosphatidyl serine and phosphatidyl ethanolamine decrease at 20 days. Lower concentration of these phospholipid subclasses at younger ages indicate that the enzymes involved in phosphatidyl serine-> phosphatidyl ethanolamine-> phosphatidyl choline conversion or cytidyl transferase are under the influence of corticosterone in young ages but not as the age advances. In this connection phosphatidyl choline synthesis is enhanced by corticosterone in lungs as it is an important component of the surfactant. On the other hand studies in liver carried out by Kaur *et al.* (1989) indi-

cate an increase in turnover of the same in liver mitochondria with dexamethasone treatment. In the present study the ^{32}P incorporation was higher in phosphatidyl serine and phosphatidyl ethanolamine and lower in phosphatidyl choline with ADX. This is consistent with the observations indicating that the pathway of phosphatidyl serine-> phosphatidyl ethanolamine->phosphatidyl choline is regulated by corticosterone.

(3) It was interesting to note that the change in concentration of these phospholipid subclasses were similar not only with dexamethasone- a synthetic analog, or ACTH- which is supposed to elevate circulating levels of corticosterone but also with testosterone- a gonadal steroid. However corticosterone and testosterone differed with respect to rate of ^{32}P incorporation in these lipids, hence may have a different effect. These results gave an impetus that like other tissues, brain phospholipids are also regulated by circulating levels of corticosterone.

(4) The next experiment was aimed to see (a) to what extent the effects of corticosterone are age dependent, (b) how do different regions which not only differ in the age at which they reach adult values, differ in the adult stage with respect to total number of cytosolic receptors as well as the proportion of type I and type II receptors. The picture emerged that amongst various class of lipids namely cholesterol, galactolipids and subclass of phospholipids, the concentration of galactolipids didn't show any effect under any conditions whereas cholesterol was the most sensitive parameter. Although effects varied with age, region studied or the treatment given the subclass of phospholipids showed very selective ef-

fects. Brain stem showed consistently low concentration of cholesterol at postnatal age 10 and 20 but remained unaffected at older ages whereas adrenalectomy increased concentration. These results clearly reveal that the cholesterol metabolism is under the regulation of glucocorticoids. The activity of HMGCoA reductase is regulated by cAMP mediated phosphorylation-dephosphorylation process, but in addition to this mode, the activity of this enzyme is also dependent on the availability of substrate and feed back regulation. The other regions also which showed only age specific effects, i.e., at postnatal day 20, were cortex and striatum and hippocampus. All the three show an increase in the levels of cholesterol but not at any other age or in ADX. In this connection glucocorticoid are known to have age specific induction of several enzymes, specifically neurotransmitter related enzymes like tyrosine and tryptophan hydroxylases between 2nd and 3rd week postnatally or after this age. From the previous study (Bhargava *et al.*, 1991)as well as the present study it appears that at least during development, regulation of cholesterol synthesis is complex .

With regard to phospholipid subclasses, the effects were apparent at younger ages. Sphingomyelin was significantly high in olfactory bulb and brain stem at day 10 and in addition to these two regions, striatum also had higher levels of sphingomyelin at 20 days of age. At older age no such effects were observed. With adrenalectomy only olfactory bulb had decreased levels whereas no other region showed any effect. Phosphatidyl ethanolamine was another subclass of phospholipid affected at 10 days of age, only olfactory bulb and brain stem. In olfactory bulb there was significantly higher concentration whereas it was lower in brain stem. At 20 days

phosphatidyl ethanolamine was lower in brain stem but was higher in striatum. Effects were not evident at older age nor ADX at this age had any effect. The other phospholipid subclasses which showed age and region specificity was phosphatidyl serine, which was significantly increased in hippocampus and phosphatidyl inositol in striatum at 20 days of age but no other changes were observed. In adrenalectomized animals the phosphatidyl choline concentration was significantly higher in brain stem.

The *in vitro* ^{32}P incorporation study was carried out only in the three regions studied namely the cerebral cortex, brain stem and the hippocampus. At postnatal age 20, elevated levels of corticosterone in both cortex and brain stem had significantly lower rate of ^{32}P incorporation in phosphatidyl inositol and sphingomyelin whereas hippocampus didn't show any effect. Adrenalectomy for different duration 5,15 and 30 indicate variability in the rates of incorporation. In the cerebral cortex, 5 days showed decreased phosphatidyl inositol incorporation,after 15 days it increased,and after 30 days it was decreased. But 30 days after adrenalectomy even phosphatidyl serine and sphingomyelin also showed a significantly lower rate of incorporation. In brain stem, the rate of incorporation increased with 5 days of adrenalectomy and decreased with 15 day adrenalectomy. Phosphatidyl choline also showed similar effects but in 30 day after adrenalectomy all these effects disappeared. Hippocampus showed only higher rate of incorporation after 15 days of adrenalectomy but the effects disappeared by 30 days.

Although results are confusing and extremely variable with age and

region, they throw light on several important points. These points are (A) The results do not concord with receptor levels nor their type. If one considers results with adrenalectomy only, it is evident that hippocampus which has not only very high number of total receptors but also has highest number of type I receptor. The type I are supposed to mediate permissive action in hippocampus. Age specific effect showed with elevated corticosterone treatment a significant increase only in phosphatidyl serine levels, whereas with adrenalectomy phosphatidyl serine increases after 15 days of adrenalectomy, and these effects disappear by prolonged condition (30 day ADX) indicating a well regulated phospholipid metabolism.

These results indicate two possibilities (i) either the phospholipid metabolism is not under the regulation of glucocorticoid in these regions or (ii) the glucocorticoid receptors are very well regulated and show up and down regulation with changes in circulating levels of corticosterone. The latter phenomenon has been demonstrated very clearly with hippocampus (Spencer *et al.*, 1991). If this is so, since phospholipids play a crucial role in regulating membrane functions, the hippocampus cells may resist any change in the concentration of phospholipid or their turnover by regulating number of corticosterone receptors. Such studies on cerebellum have not been reported.

(B) The phospholipid metabolism is an extremely dynamic process and several factors seem to regulate/influence the metabolism of the same. The dynamicity can be observed from the fact that elevated levels of corticosterone only affect at younger ages and adrenalectomy of different durations show different effects on the rate of incorporation of ^{32}P. They

more or less overcome the effects with longer duration of adrenalectomy. Only cerebral cortex seems to become more sensitive with prolonged adrenalectomy. The bimodel responses have been observed in cAMP levels with different duration of dexamethasone treatment (Duman *et al.*, 1989).

(C) The striatum seem to be unique in the sense that at younger ages, elevated levels of corticosterone show relatively greater effects. The possible reason could be that striatal nuclei seems to show greater retention of corticosterone, perhaps enhance gene expression.

(D) Of all the regions studied brain stem lipid metabolism seem to be regulated very similar to what has been observed in the peripheral component, whereas rest of the regions show differential effects.

(E) Since concentration of phosphatidyl inositol in the cortex was not affected but changes in the rate of ^{32} P incorporation was observed at all ages with elevated or depleted status emphasizing phospholipase-C mediated effects. So phosphatidyl inositol metabolism in the cortex is a major factor.

(5) The next experiment was aimed to check fatty acid composition of phospholipids subclass. However due to limitation in the facility available and time alloted by the institute which permitted this assay it was possible only to study the synaptosomes prepared from cerebral cortex of 20 day old control and corticosterone treated animals. The study revealed that although concentration was not changed, there was significant changes in the proportion of unsaturated fatty acids especially in phosphatidyl inositol, phosphatidyl serine and phosphatidyl ethanolamine. There was a shift

from saturated to unsaturated levels. 20:4 increased in phosphatidyl inositol and phosphatidyl ethanolamine, 22:6 in phosphatidyl serine and in phosphatidyl choline fatty acids were not altered. Thus indicating an increase in desaturase activity and increase in replacement of unsaturated fatty acid at C-1 position in phosphatidyl inositol. These results indicate that the fatty acid synthesis and their incorporation in phospholipids seem to be one of the factor which is under the influence of corticosterone. In this connection the enzymes such as fatty acid synthase and acetyl CoA carboxylase involved in fatty acid synthesis are known to be regulated by glucocorticoid in tissues other than brain (Battenburg and Elfring,1992).

(6) Changes in the cholesterol concentration, rate of ^{32}P incorporation and alteration in specific phospholipids subclass in different region, especially profile of fatty acids in the phospholipids fractions from cerebral cortex led to the next experiment to investigate membrane bound enzymes important for neurotransmission, Na+K+ATPase, 5' nucleotidase and acetylcholine esterase. The crude membrane were used in this study to avoid any cytosolic or extracellular factor influencing the activity. The study revealed that the Na+K+ATPase was the most sensitive whereas AchE the least and 5 'Nucleotidase activity was altered only during early ages with corticosterone treatment. Na+K+ATPase was also affected with adrenalectomy. The pattern of changes in activity was uniform in all three regions irrespective of the stages of maturation of the regions and corticosterone receptor number. Na+K+ATPase was found to be increased at 10 days in hippocampus, striatum and cortex. In addition to these regions

cerebellum also shows increase 5' nucleotidase activity while AchE was significantly increased only in striatum.

At 20 days of age both enzymes showed lower activity in treated group, however 5' nucleotidase was affected in selective regions namely cerebellum and striatum. The effects were also evident in synaptosomes isolated from cortex and cerebellum Na+K+ATPase and 5' nucleotidase activity. The latter enzyme was affected only in myelin of cerebellum. Adrenalectomy (15 days duration) at younger age had significantly increased Na+K+ATPase activity in most of the regions, however adrenalectomy for prolonged period (5 months) in aged animals didn't show any effect. Thus either the age or prolonged condition reversed the effects. The alteration in enzyme activities which are integral proteins have requirement of specific lipids. Two possible factors could alter the activities of isolated membranes.

(i) Genomic effect on the synthesis of the protein of Na+K+ATPase or (ii) due to alteration in the lipid environment which could influence the activities. In this connection, it is known that polyunsaturated fatty acid inhibit Na+K+ATPase and in the present study increase in proportion of polyunsaturated fatty acid was observed in synaptosomes

(7) The changes in synaptosomal phospholipid fatty acid composition and membrane bound enzymes led the next experiment to examine the fluidity. The fluidity was found to be similar to control as judged by DPH probe. The anisotropy and microviscosity were similar at 37°C and 25°C in the synaptosomal membranes from corticosterone treated and control group, however

fluidity was significantly decreased at 0° C in corticosterone treated groups. Since such low temperature has no physiological relevance it indicates that the membranes components have altered and these alteration are to maintain the membrane fluidity at these temperature relevant at physiological condition. In this connection *in vitro* addition of cortisol in the synaptosomes isolated from dog cerebral cortex was found to increase the fluidity (Deliconstatinos, 1985). The results obtained in the present study indicates that the changes in cholesterol and the phospholipid concentrations/turnover/fatty acid composition is to maintain membrane fluidity.

(8) Additional studies were carried out on the sensitivity of the young and aged animals and adrenalectomized animals by studying the morphology of hippocampal cells,since hippocampus is the target region for corticosterone effect. The biochemical parameters studied revealed that sensitivity of hippocampus changes with age.

Histological studies revealed granule cell loss depends on age as it is higher in younger animals but in aged animals it was not conspicuous. The extent of loss even in young animals was not as spectacular as reported. The possible factor could be that in the present study the animals were handled from birth till the age of termination of the experiment and handling seems to retard aging.

The pyramidal cells of the CA_3 region are sensitive to excessive levels of corticosterone which normally increases with aging. Hence, in aged animals, cells of CA_3 have altered morphology and increased cell death resulting in increased gliosis which adrenalectomy prevented.

In conclusion this is the first systematic attempt to investigate the phospholipid concentration and its turn over in different brain region in relation to circulating levels of corticosterone at different ages. The results indicate that phospholipid subclasses are under the regulatory influence of corticosterone. These effects are more related to age rather than the receptor density or types. It appears that the corticosterone influence the enzymes associated with phospholipids subclasses at the transcriptional level at younger ages, while the phospholipase C especially, phosphoinositides sensitive is under the regulation of corticosterone at all ages. The type of fatty acids incorporated or exchanged seems to be one of the means of alterations in membrane constituents. The effects of membrane bound enzymes seems to be the consequence of alterations in the membrane composition. In spite of the changes in the membrane constituents the membrane fluidity is maintained at 37°C and 25°C and this reflects the membrane integrity is maintained in the event of differing levels of corticosterone.

These results throw light to the mechanism of coping with an intruder which can enter the cell without requiring any specific machinery for entrance. It can not only enter but can also interfere simply getting intercalated with itself in the membrane. Since experiments involve adrenalectomy procedure, it is likely that some of the steroid remain intercalated for a longer duration than 24 hours. In the present studies experimental animals were killed for biochemical studies 24 hours after the last injection.