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Dr. B.K. Anand Oration

Hypothalamic Regulation of Energy Homeostasis

Neurophysiological mechanisms underlying obesity and diabetes mellitus

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Preamble :

Although the story of Dr. Anand's discovery of a set of neurons in lateral hypothalamus involved in feeding behaviour is known to several investigators, it is perhaps worth recapitulating so as to enable the younger biomedical scientists to share the excitement of the events of November, 1950 when Dr. Anand joined Prof. Brobeck in Prof. Fulton's Lab at the Yale University School of Medicine. Having observed a few times the technique of stereotaxy as used by Brobeck, Anand wished to repeat Brobeck's experiments in which lesions of ventromedial hypothamalus (VMH) resulted in hyperphagia and obesity in the rat (1). He followed the same procedure with meticulous precision in 6 rats, but the animals, instead of getting hyperphagic and obese, refused to eat even if food was placed in their mouth. They lost weight and died in a few weeks time.

On autopsy, it was found that rather than well circumscribed discrete lesions in the VMH as produced by Brobeck, Anand's experimental rats had 'giant' lesions in the hypothalamus, destroying both ventromedial as well as a part of lateral hypothalamus. Looking back, Anand wondered as to what had gone wrong. He had precisely followed the same procedure and there was nothing wrong with his technique. It was then found that there was a malfunction of the instrument and instead of delivering a current of 2 milliamperes, the instrument delivered a current of 20 milliamperes, thus destroying a much larger area which included both VMH and lateral hypothalamus (LH). With Sheringtonian logic, Anand, with his brilliant intellect and incisive mind hypothesized that big lesions also destroyed the neurons in the lateral hypothalamus which were possibly responsible for initiating feeding behaviour.

Experimental evidence was soon generated to support this hypothesis by producing discrete lesions in the lateral hypothalamus. The destruction of these neurons led to a complete cessation of feeding by the animals. Anand, with Brobeck, in their paper in February, 1951 reported that a small area had been

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localized in rats in the extreme lateral part of the lateral hypothalamus, in the rostrocaudal plane (2). Bilateral destruction of this area resulted in a complete cessation of eating. A unilateral lesion had no such effect. This lateral area was designated a "feeding centre" (2). It was considered to be responsible for central hunger reaction or the urge to eat. As they say, the rest is history.

The second epoch-making publication by Anand and Brobeck appeared in November, 1951 and needs to be cited verbatim : 'It is thus clear from these operations that bilateral destruction of the lateral portion of the lateral hypothalamus, in the same rostro-caudal and horizontal planes as the ventromedial nucleus, abolishes the food intake of rats, even in animals which have previously been made hyperphagic and obese. None of the animals in which this area of the lateral hypothalamus has been bilaterally destroyed has ever eaten any food during entire period of its postoperative survival- and this in spite of our many attempts to put food near to or even inside the mouth' (3). These significant observations set the tone and pace for subsequent work in this area. The following decade generated active interest in this field and supportive evidence came through several studies, some of which were conducted in Anand's lab after he joined AIIMS in 1956 (4).

The next landmark was the work based on the recorded activity of single neurons from VMH and LHA. An increase in the firing rate of neuron units in the VMH in the fed state was demonstrated, along with a corresponding decrease in activity from LH in such a satiated state. On food deprivation, there was a reversal of single unit activity in these areas with an increase in the firing rate of neurons in the LH. Thus a reciprocal relationship between the activities of neurons in VMH and LHA was firmly established. More importantly, these studies also provided the basis for postulated 'glucoreceptor' neurons in these hypothalamic centers (5). The data published in the paper in Am. J. Physiol. is now considered a classic in the neurophysiology of feeding behaviour (6).

This was the state of art in 1966-67. There existed overwhelming evidence for hypothalamic regulation of feeding behaviour. It is at this time that I initiated collaboration with the group of Prof. G.S. Chhina and Prof. Baldev Singh in the department of physiology where several young doctoral and postdoctoral students, including Dr. V. Mohan Kumar were involved in active research.

In view of my deep interest in the study of basic mechanism in diabetes, a chronic diabetic state was produced in the rhesus monkey with I.V. injection of Streptozotocin, which results in extensive necrosis of pancreatic β-cells. Biopolar EEG recordings showed a slower activity in the VMH and a faster activity in the LHA after induction of experimental diabetes. EEG activity from other areas such as pre-optic and cerebral cortex remained unaltered in these animals (7). Following insulin administration, these EEG abnormalities were reversed. In a series of publications, it was concluded that it was not the amount

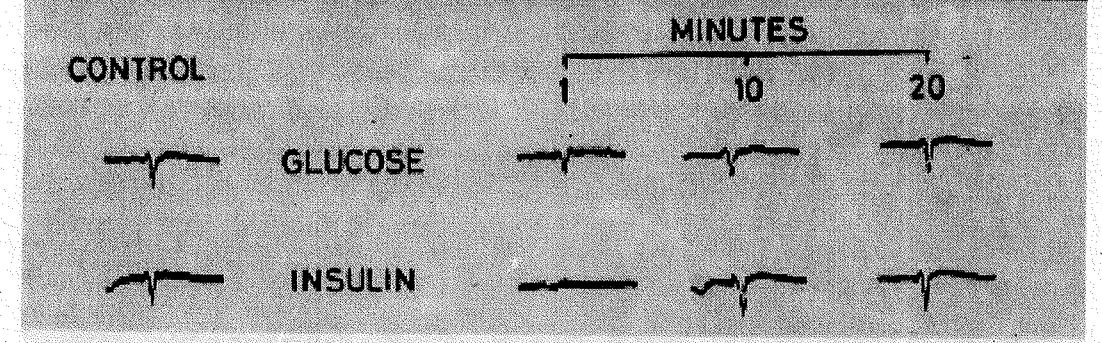
of glucose reaching hypothalamus, but its *net* utilization by these neurons that reflected their activity. For the first time, sound experimental evidence had been generated to explain the possible pathophysiological basis of hyperphagia in human diabetes.

In collaboration with Dr. Mohan Kumar, it was also shown in male cats that evoked responses from VMH and LHA, following stimulation of mesenteric nerves, were modified by I.V. injection of glucose or insulin. Administration of glucose produced initial decrease in amplitude, which gradually recovered while insulin produced an initial short acting inhibition followed by an increase in amplitude (8). (Fig. 1) These studies once again reemphasized the relationship between net rate of neuronal glucose utilization as an important determinent of the amplitude of evoked responses from VMH and LH.

In the late 60s and early 70s, the standard textbooks of physiology stated that insulin does not affect glucose metabolism of the brain, and that insulin does not cross blood-brain barrier. However, the data generated at the AIIMS had convincingly shown the effect of glucose and insulin on the activity of neurons in hypothalamus, and emphasized that the glucose utilization of neurons in VMH and LHA was a determinant of the neuronal activity (9).

The next logical question was : Is there a hypothalamo-insular axis? It was hypothesized that as insulin influenced the activity of VMH and LH neurons, these

Fig. 1 : Effect of glucose and insulin on ER from LHA

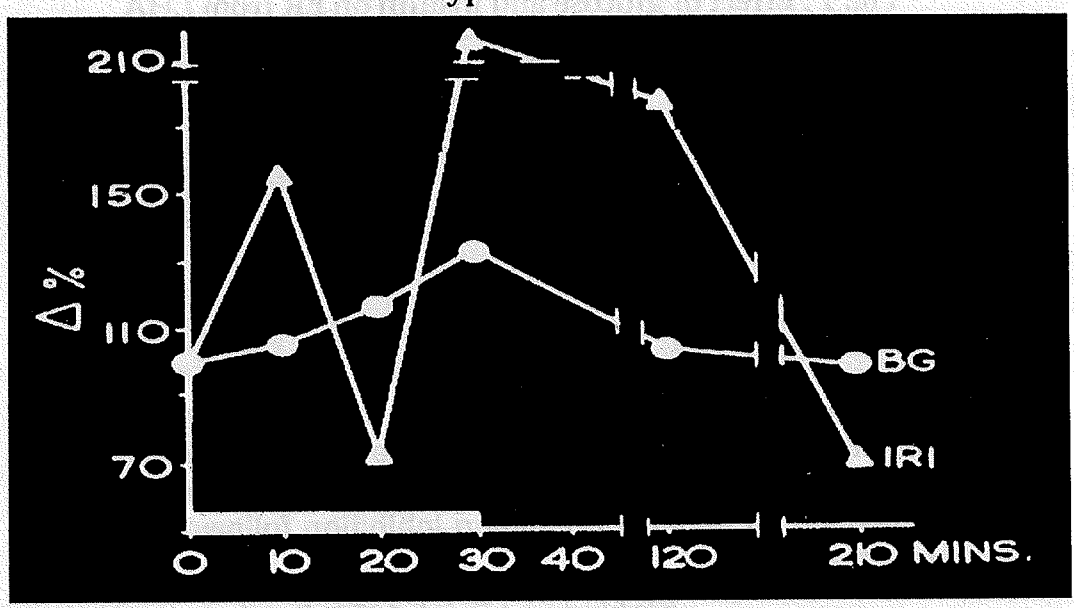


Effects of glucose and insulin on ERs from lateral hypothalamus on stimulation of mesenteric nerves. Glucose produced initial drop in amplitude which gradually recovered and then even increased. Insulin caused an initial short-lasting inhibition followed by an increase in amplitude.

(Chhina and Bajaj, 1972; Bajaj et al., 1975)

neurons may likewise affect insulin release from pancreatic β-cell. With SK Garg, a doctoral student, and under an ICMR grant to the author as Principal investigator, the effect of stimulation of different areas of brain on feeding behaviour and on circulating levels of insulin was studied (10). As observed earlier, stimulation of LHA increased food intake, while VMH decreased intake of food. More importantly, stimulation of LH in conscious restrained monkeys increased serum insulin while stimulation of VMH decreased the levels of circulating insulin. (Fig. 2) Based on the experimental data, Bajaj and Chhina proposed in 1976 the existence of Entero-hypothalamo-insular axis. It was conceptualised that in the fasted state, reduced activity in the neurons of the ventromedial hypothalamus with a reciprocally increased activity in those of the lateral hypothalamus would result in the initiation of feeding behaviour providing a neural or neurohumoral signal to the β-cell, and initiating what may be termed as the cephalic phase of insulin release. Intestinal motility, blood flow and rate of nutrient absorption would also be altered through the autonomic responses. Food intake would also result in the release of gastrointestinal hormones (*presently* called *Incretins)*, some of which will further increase insulin secretion from the β-cell. Finally, a rise in blood glucose will directly stimulate the glucose receptor in the β-cell, thus further increasing the levels of circulating insulin and enhancing glucose utilization in the body as well as in the hypothalamic neurons. Presence of food in the intestines and increased glucose

Fig. 2 : Δ Blood glucose & serum insulin : Lateral Hypothalamus stimulation



BG = Blood glucose; IRI = Immunoreactive insulin

utilization by insulin release provided afferent inputs initiating satiety behaviour and cessation of food intake (7,11).

Glucose sensitive neurons in VMH : Supportive Evidence

In addition to the evidence provided through studies referred to above, supportive evidence was generated by other investigators using mice that had been injected with gold thioglucose. As is well known, gold is toxic to neurons and leads to their destruction. When gold is coupled to glucose by sulphur (aurothioglucose : ATG; auro, gold; thio, sulphur) and injected in the mice, extensive damage is done *only* to those neurons in VMH which take up and metabolise glucose. Following the injection of ATG, mice become hyperphagic within 15 hrs. followed by continuing hyperphagia that subsequently leads to obesity (12). Interestingly, such chemical lesions cannot be produced when other goldthio-compounds are injected (13). Furthermore, damage to the VMH is minimized if mice are injected with 2-deoxyglucose (2 DG) prior to the injection of ATG as the former blocks 'glucoreceptors' (14). Likewise, the damage is minimal if ATG is injected in animals previously made diabetic (15). On the contrary, the damage to VMH by ATG is greater if mice are injected with insulin prior to the administration of ATG (16). Subsequent studies provided further support as ATG-induced damage was prevented by the administration of betathioglucose, an antimetabolite of glucose (17). Similarly, administration of phlorizin, which inhibits glucose transport, prevented ATG induced hyperphagia and obesity (18).

Point : Counter Point

The chronology of this line of research dated from late 60s and extended into late 80s. It provided strong support for the hypothesis generated by us, which in turn was based on early work by Anand and his coworkers, using entirely different experimental design and research methodology. Nevertheless, as in any scientific and intellectual pursuit, counter challenges were mounted by a number of investigators, principally pioneered through a paper published in 1974 by Richard Gold entitled "Hypothalamic obesity : the myth of the ventromedial nucleus" (19). Even prior to this publication, doubts were being expressed regarding the precise location of target neurons exclusively in the VMH to warrant its recognition as 'satiety centre'. In a series of experiments, lesions limited to the VMH in guinea pigs of either sex failed to produce anticipated excessive weight gain. Indeed when Rosen, a doctoral student, failed to produce hyperphagia with VMH lesions in the male rats (20), she concluded : 'The production of obese rats by neurological damage must be an art rather than a science'.

Additional studies of lesions produced with ATG in mice hypothalamus showed initial changes localized in the area ventral to VMH, and including the arcuate nucleus (ARC), the damage later spreading to VMH (21). These observations questioned the role of VMH as the exclusive site which when damaged resulted in production of hyperphagia and obesity. The new experimental data suggested that damage to ARC may also play a significant role.

While these studies doubted the exclusive role of VMH in the neuroregulation of satiety behaviour, Gold's research in female rats convincingly demonstrated that electrolytic lesions restricted *only* to VMN produced neither hyperphagia nor obesity. Such lesions cause obesity *only* when they extend *beyond* VMN. The magnitude of obesity was related to the extent of area damaged *adjacent* to VMN (22). It was hypothesized that obesity observed in rats with lesions extending just rostral to VMH was due to damage to the ventral noradrenergic bundle (VNAB). Lesions of the VNAB at the level of midbrain cause a significant reduction in norepinephrine in hypothalamus, thus resulting in hyperphagia and obesity (23). Several investigators confirmed Gold's observations and reported that long parasagittal knife cuts extending laterally to anterior hypothalamus were more effective in producing hyperphagia and obesity (24). Finally it was shown that although electrical stimulation of VMH suppressed feeding in food deprived rats, parasagittal knife cuts between VMH and LH failed to prevent the stimulation-induced inhibition of feeding. What sounded like a requiem for the VMH was the observation by Leibowitz et al. that lesions of paraventricular nucleus (PVN), *with no damage to VMH*, resulted in hyperphagia and obesity in rats (25).

As if these counterarguments were not enough to alter noise : signal ratio in this cacophony, evidence was generated that obesity resulting from VMH lesions was metabolic in origin, and not primarily as a result of hyperphagia. Weanling rats given VMH lesions do not display hyperphagia or excess weight gain but develop marked increase in body fat content (26). The pathophysiological basis for this was considered to be hyperinsulinemia in nonhyperphagic weaning rats with VMH lesion (27). Subsequently, hypothalamic obesity was shown to be reversed by subdiaphragmatic vagotomy (28). It was considered that VMH obesity was the result of an increase in all vagally mediated (parasympathetic) reflexes and that hyperphagia was secondary to such alterations in visceral metabolism.

Resurgence of Interest in VMH :

The revival of interest in the role played by neurons in the VMH (and LH) is due to the availability of newer techniques of molecular biology which have facilitated our understanding of the role of autonomic nervous system and neuroendocrinal regulation of energy balance (29). There is now substantial evidence that the VMH plays a key role in generating and regulating autonomic responses, both parasympathetic and sympathetic, and that any alterations of these responses through VMH lesions contribute to obesity. The consensus seems to be that VMH lesions result in a metabolic disorder leading to obesity, *in addition* to causing hyperphagia which is possibly independent of metabolic events and which also contributes to the causation of obesity.

Presently, there is a general consensus that a physiological system maintains homeostasis of energy stores in response to

varying availability of food and changing demands of energy expenditure. The energy demands of the body under resting basal, active and stressful conditions are adequately and appropriately responded to in a short period of a few minutes, by glucose which constitutes a most dependable energy supply source on a short term basis, although in the long term, body adipose tissue responds to the needs for the maintenance of energy balance. The evidence for the neuroendocrinal regulation of energy balance had been previously reviewed and it was stated : 'the rate of glucose utilisation seems to be the set point in the regulation of entero-hypothalamo-insular axis. However, this may be so for the maintenance of energy balance on a short term basis. Adipose tissue functions as the major source of energy fuel; during starvation, glycogen stores in the human body may sustain life for less than 24 hours while energy stored as triglycerides can maintain supplies to vital organs for 30-60 days. It is therefore possible that control of triglyceride storage may be of considerable influence as a long range regulator of entero-hypothalamo-insular axis' (8).

Hypothalamic Regulation of Energy Homeostasis :

The subject has been recently reviewed (29) and relevant excerpts are cited here so as to maintain narrative continuity. The hypothalamus is the principal brain region that acts as a key determinant in the integrated control of feeding, energy homeostasis, and regulation of body weight. Hypothalamus senses neural, endocrine, and metabolic signals, integrates these inputs, and engages distant effector pathways, resulting in behavioural, autonomic, and endocrine responses (30). The hypothalamic control and regulatory mechanism is mediated through a complex array of neuroendocrinal signaling pathways involving synthesis and release of several neurotransmitters and neuropeptides. These include monoamine neurotransmitters, such as 5-hydroxytryptamine, norepinephrine, as well as orexigenic neuropeptides (neuropeptide Y, orexins A and B) and anorectic peptides (cocaine-and amphetamine-regulated transcript: CART; pro-opiomelanocortin : POMC and related peptides i.e. αMSH) (31). A brief perspective of the role and action of these peptides is provided in the following paragraphs.

The positional cloning of ob gene in 1994 (32), and the subsequent discovery that the encoded protein, named leptin, functions as an adipocyte derived signal for the regulation of feeding behaviour, set the direction and pace of research during the last decade. Ob receptor gene was cloned a year later, in 1995 (33), and leptin receptors were demonstrated in the arcuate nucleus (ARC) of hypothalamus (34). ARC is located in the mediobasal hypothalamus adjacent to the floor of the third ventricle. It contains neurons that respond to afferent signals, predominately hormonal, which reflect and relate to the *size* and *state* of adipose tissue stores. Although leptin is secreted primarily from adipocytes and insulin is released from the endocrine panacreas, both

circulate at levels proportionate to body fat mass and exert relatively long-lived inhibitory effects on food intake via actions on their receptors in the ARC. These actions are mediated through a set of neurons in the ARC which coexpress neuropeptide Y (NPY) and agouti-related peptide (AgRP). These two peptides are potent stimuli of food intake; moreover, these peptides also reduce energy expenditure and thus promote weight gain. In contrast, the ARC also contains neurons that synthesise αMSH (Melanocyte stimulating hormone) that exert a powerful anorectic effect. αMSH is synthesized from its precursor proopiomelanocortin (POMC). Many POMC neurons also coexpress another peptide called CART (cocaine and amphetamine related transcript). Both αMSH and CART reduce food intake. α-MSH is an agonist for melanocortin 4 receptor (MC4R) which also appears to be involved in the regulation of appetite and body weight mediated through 5-hydroxytryptamine receptors, activation of which causes weight loss and deletion of which causes adult-onset obesity in mice (35).

Insulin and leptin signals, which are enhanced in a state of excess adipose tissue mass i.e. obesity, are inhibitory to NPY and AgRP neurons and facilitatory to POMC and CART neurons, the net effect of these hormones results in inhibition of feeding behaviour (36). It is through this reciprocal regulation of anabolic and catabolic neuronal circuits that insulin and leptin mediate their effects on energy balance. It was hypothesized that a decrease in plasma levels of insulin and leptin which follows a reduction in body fat mass, results in the activation of NPY/AgRP and inhibition of POMC neurons.

Insulin and Hypothalamus :

Insulin functions not only as a peripheral regulator of nutrient storage and release of circulating substrates but there is increasing evidence that in the brain, insulin is involved in a wide array of regulatory mechanisms including neuronal survival, neuronal plasticity, learning and memory, as well as energy homeostasis and reproductive function.

Essentially, glucosensing neurons are predominantly located in those areas of brain that are involved in the control of neuroendocrine function, nutrient metabolism, and energy homeostasis. A select group of such neurons use glucose as a signaling molecule to alter their firing rate both as a means of, and also as a response to, glucosensing. The VMH contains both the ventromedial hypothalamic nucleus (VMN) and the arcuate nucleus (ARC). Both contain glucosensing neurons that respond to changes in ambient glucose levels. Through effector pathways, these neurons are involved in regulation of glucose homeostasis. The population of glucosensing neurons in the VMH (VMN & ARC) is amongst the best characterized with respect of glucosensing. In the VMN, 14-19% neurons are glucose-excited (GE) and 3-14% are glucose-inhibited (GI) in type (37). Glucosensing neurons use glucose in a concentration dependent manner as a signaling molecule to regulate their membrane potential and action potential

frequency (38). It has been suggested that GE neurons are analogs of pancreatic β-cell, whereas GI neurons have some similarities to pancreatic α-cells : GE neurons and β-cells are activated and GI neurons and α-cells are inhibited by increase in ambient glucose levels (39) . The LH contains predominantly glucose-inhibited neurons (40).

Insulin Signaling in Hypothalamic Neurons

Glucokinase (hexokinase IV) is a key regulator of neuronal glucosensing, thus performing a role similar to that in the pancreatic β-cell (and α-cell) glucosensing (41). In the ARC, more than 75% of Neuropeptide Y-(NPY-) positive neurons express glucokinase (42). Many glucokinase-expressing neurons coexpress K_{ATP} channels (43). Furthermore, coexpression of GLUT-3 and GLUT-4 with insulin receptor mRNA (IR mRNA) is also reported in glucose-responsive neurons (44). Recent studies confirm that glucose-excited neurons utilize ATP-sensitive K^+ channels as their transduction mechanisms for glucosensing whereas glucose-inhibited neurons appear to utilize a nonspecific Cl^- channel. Irrespective of the type of ion channels used as a final common pathway, a large proportion of glucose-excited and inhibited (GE and GI) neurons appear to utilize glucokinase as a regulator of glucosensing. Glucokinase mRNA is selectively localized in several brain areas involved in glucosensing. It is expressed in ~ 70% of GE and ~ 40% of GI neurons (44). Final confirmation of the key role of neuronal glucokinase is based on the fact that a knockdown of glucokinase mRNA, using glucokinase siRNA in primary hypothalamic neuronal cultures, ablates the ability of these neurons to sense glucose (45).

Protein Tyrosine Phosphatase 1B (PTP1B) has been shown to be a negative regulator of insulin signaling by dephosphorylating key tyrosine residues within the regulatory domain of the β-subunit of the insulin receptor. Recent gene knockout studies in mice ($Ptpn1^{-/-}$ mice) have shown an increase in insulin sensitivity in such animals; these mice are lean and show resistance to high-fat diet-induced obesity (46,47). Studies using Ptpn1 antisense oligonucleotides, which lower PTP1B levels only in liver and adipose tissue, tended to suggest these tissues as the main sites of action for the regulation of glucose homeostasis and lipid metabolism. A recent study with tissue specific deletion of PTP1B in brain, muscle, liver or fat has shown that neuronal PTP1B also regulates insulin sensitivity as well as degree of adiposity. Indeed mice lacking PTP1B in brain show enhanced peripheral insulin sensitivity accompanied by increased insulin receptor phosphorylation in muscle and liver. Involvement of neuronal pathway controlling hepatic glucose production or affecting adipokine secretion could possibly be underlying contributory factors.

Realising the therapeutic potential of inhibiting PTP1B in promoting weight reduction and improving insulin sensitivity and glucose homeostasis, efforts have been intensified in developing inhibitors of PTP1B which may be effective in treating insulin resistance at an early stage, thereby preventing Type 2 diabetes mellitus (T2DM) and obesity (48).

Insulin Signaling in CNS : Evolutionary Perspective

Insulin signaling in neuronal cells plays a key role not only in mammals but also in primitive organisms such as the nematode *Caenorhabitis elegans* and the fruit fly *Drosophila melanogaster* (49). Indeed, insulin signaling pathways show several similarities in *C.elegans, D.melanogaster*, rodents, and humans, thus raising the distinct possibility of an evolutionary mechanism conserved over the millennia. Neurosecretary cells in *D.melanogaster* express insulin-like peptides (dILPs). Ablation of dILP neurons results in prolonged life span, reduced fertility, increased fasting glucose levels, increased storage of lipids and carbohydrates, and reduced tolerance to heat and cold, thus highlighting the key role of these cells in the regulation of life-span and fuel metabolism (50).

Additional discoveries in this connection have clarified the role of insulin signaling in providing metabolic connectivity between nutrition, reproduction and lifespan. The initial discovery in *C.elegans* was the cloning of *DAF-2,* the gene that encodes a homologue of the mammalian insulin receptor, containing both ligand binding and tyrosine kinase domains (51). The relevance of DAF-2 to *C. elegans* physiology was initially based on its association with a stage of diapause arrest called "dauer." The *dauer* is characterized by inhibition of reproduction and reduced metabolism and growth, thereby resembling suspended animation or hibernation. Such a state is normally triggered by periods of reduced food availability. Mutations of DAF-2 were shown to produce the dauer state and also revealed DAF-2 as the first step in a signal transduction cascade homologous to the insulin pathway described in mammals. One of the proteins in signal transduction pathway is called advanced glycation end product-1 (AGE-1), a homologue of mammalian phosphatidylinositol 3-kinase (PI 3K). The knockout of PI 3K induces a phenotype dauer stage, which is characterized by increased longevity as is seen with mutation of DAF-2 (51). Another key protein is DAF-16, a member of the forkhead transcription factor family related to mammalian HNF-3 and FOXO1. The finding that DAF-16 mutation completely reversed the phenotype arising from DAF-2 or AGE-1 knockout (52) (a phenomenon referred to as genetic complementation) suggests that this forkhead protein functions downstream of the more proximal DAF-2 and AGE-1 proteins, and that its activity is normally inhibited by activation of the upstream DAF-2/AGE-1 cascade, and finally that this inhibition is a dominant component of signaling in this cascade (52).

It was suggested that increased longevity associated with the DAF-2 knockout is analogous to the effect of caloric restriction to increase mammalian longevity, since calorically restricted animals experience decrease of both circulating insulin (and hence reduced insulin receptor signal transduction) as well as of fertility. Restoration of DAF-2 in neurons was sufficient to restore lifespan and normal phase of reproduction of DAF-2 knockouts to wild-type values, and neuron-specific restoration of AGE-1 in

animals that otherwise lack this protein produced the same effect. Thus, neuronal insulin-like signaling appears to be a key regulator of various critical functions in *C. elegans*. The metabolic and reproductive defects induced by whole-body deletion of DAF-2 appear to be due in large part to abnormalities specific to the absence of neuronal insulin-like signaling.

It is of interest to note that as of March 2002 (53), *Drosophila* gene sequences have been found with highly significant ($P < 10^{-10}$) matches to 75% of the human disease loci examined. It is amazing, therefore, that fully 75% of human disease loci have counterparts in *Drosophila*. While the insulin-like receptor was first reported in this species in 1996, knockouts were found to be lethal; hence, insulin receptor-like activity is absolutely essential for life during development. However, mutations of either an Insulin Receptor Substrate (IRS) homologue termed *CHICO*, or complex heterozygotes of the insulin-like receptor, were shown to extend lifespan and reduce reproduction in a manner similar to that induced by DAF-2 mutants in *C. elegans* (54). As in *C. elegans*, lifespan extension was associated with a general growth deficiency and a decrease in cell number and size, and insulin-like signaling was shown to depend on a PI3K homologue.

Some evidence of the existence of insulin-like peptides exists in plants as well. As early as 1960, extracts of the plant *Momordica charantia linn* (bitter gourd) were shown to elicit a hypoglycemic response (55). A polypeptide was subsequently partially purified (56). After labeling with ^{125}I and further purification, this peptide was subjected to additional immunological studies in our laboratory. It was found that the material did not cross-react with anti-insulin serum. In contrast, application of wick chromatography, a technique that we had earlier found to be of value in identifying basic and acidic polypeptides (57), seemed to suggest that the hypoglycemic plant extract was an acidic polypeptide, with behaviour similar to that of the A-chain in insulin. Of particular significance is also the finding that insulins of vertebrates share a common gene lineage and have a highly conserved sequence *Gly-Phe-Phe-Tyr* (residues 23-26) in the B-chain, which constitutes a critical area of the receptor-binding region of the molecule. Our group did theoretical calculations on the minimum energy conformation (three-dimensional structure) of this conserved sequence, using a global optimization technique developed by Subba Rao et al (58). Results show that this conserved sequence has a specific conformation that gets significantly altered if either of the two *Phe* residues is substituted by *Leu* or *Ser*, resulting in a decreased activity of insulin (59). Such substitutions have been identified in a few families with hyperinsulinemia and T2DM and have been designated as insulinopathies.

On the basis of evidence presented thus far, it may be rationally argued that an early evolutionary role for insulin may have been to regulate metabolism through neuronal control of nutrient storage, a process tightly coupled with control of reproduction and lifespan, since both energy storage and reproduction depend upon nutrient availability. According to this hypothesis, the emergence of insulin as a key regulator

of carbohydrate metabolism in vertebrates was a more recent evolutionary development (60). Extending the concept of evolutionary perspective with competitive survival as the key, the intimate relationship between immune and metabolic responses also needs to be highlighted. It is well recognized that functional structures that control key metabolic and immune functions have evolved from common ancestors. An oft-quoted example is *Drosophila* fat body, which contains the mammalian homologues of liver, the hematopoietic system, and other related immune components. It has been recently shown that this site also corresponds to mammalian adipose tissue (61). As these specialized cells differentiate into distinct functional units or organs, they carry with them their developmental lineage. Hence, it is possible to envision a scenario where common pathways regulate both metabolic, reproductive and immune functions through the utilization of common key regulatory molecules such as glucose and fatty acids. It is of interest to note that glucosensing neurons in VMN, ARC and LHA also respond to a variety of metabolites such as lactate, ketone bodies and free fatty acids (62,63). Long chain acyl-CoA also activates K^{ATP} channel in these neurons (64) and inhibits GK activity (65). Thus glucosensing neurons indeed function as ***metabolic sensors***. Such a closely linked configuration and coordinated regulation of metabolic and immune responses is likely to be advantageous, since the organism needs to organize and redistribute its metabolic resources during stressful life situations which require urgent mounting of an immune or inflammatory response.

The Endocannabinoid System : From Anand to Anandamide

The major discovery of the last decade regarding the existence of the endocannabinoid system has a most profound physiological impact and holds a great therapeutic potential. This discovery is centered around the well known plant, *cannabis sativa or cannabis indica*. The plant grows wild all over India (and other parts of the world) and its flowering tops, resin from tops, and leaves have been extensively used for many centuries.

Marijuana cigarettes are prepared from the leaves and flowering tops of the plant while *Hashish* is prepared from concentrated plant resin. While smoking is the most common mode of use, the oral intake of *Bhang* as a concoction is practiced in several socio-religious groups, more so during festivals. As a part of folk-lore medicine, cannabis has been used over millennia for disorders as varied as joint pains and epileptic convulsions. William O' Shaughnessy brought this substance to the notice of western medicine in the middle of the eighteenth century, highlighting the 'remarkable increase of appetite' as a result of cannabis consumption (66). It was only in 1964 that its active psychoactive constituent Δ^9-tetrahydrocannabinol (Δ^9-THC) was isolated, its structure identified, and partial synthesis accomplished (67). Δ^9-THC alongwith other naturally occurring and synthetic cannabinoids, bind with two separate G protein-coupled receptors. Cannabinoid receptor 1(CB_1) (68) is localized in the CNS including neurons in

lateral hypothalamus, and in the periphery especially in the liver and adipose tissue. In contrast, cannabinoid receptor 2 (CB_2) is found primarily on cells of the immune system (69).

A complex biochemical pathway for the synthesis, release, transport, and degradation of endocannabinoids alongwith their receptors CB_1 and CB_2 constitutes a new signaling system termed the 'endocannabinoid system' (70). Since the discovery of fatty acid amide, arachidonoylethanolamide, by Devane et al in 1992 (71), who also named it as '***Anandamide***' from the Sanskrit root 'Ananda', meaning 'internal bliss', more than 3500 scientific reports have been published exploring diverse aspects of endocannabinoid system. Essentially, evidence has been generated during the last decade indicating this signaling system as a modulator of physiological functions not only in the central nervous system, but also in the autonomic nervous system, neuroendocrinal network, the immune system, the gastrointestinal tract, the reproductive system both in the male and the female, and in microcirculation.

The two most studied endocannabinoids include Anandamide and 2-arachidonoylglycerol (2-AG). Anandamide is formed by the cleavage of a phospholipid percursor, the N-arachidonoyl-phosphatidylethanolamine (NAPE). Anandamide acts as a retrograde messenger at presynaptic cannabinoid receptor CB_1 where it regulates neurotransmitter(s) release. Anandamide also acts as a neuromodulator at postsynaptic cells where it regulates excitability. Anandamide action is terminated through a two-step process that includes i) transport into cells through a specific anandamide transporter (AT), and ii) enzymic degradation by cleavage to arachidonic acid and ethanolamide by membrane bound enzyme, fatty acid amide hydrolase (FAAH) (72), which is widely distributed in the body with high concentrations in the brain and in the liver.

Cannabinoid receptors :

A reference has already been made to the two cannabinoid receptors, CB_1 and CB_2. CB_1 receptor is mainly located in the terminals of nerve cells including central neurons in the hypothalamus and other areas of brain (73), as well as in the peripheral neurons such as those in the autonomic nervous system. CB_1 receptor has also been demonstrated in the liver (hepatocytes) (74) and the adipose tissue (adipocyte) (75). CB_1 expressed in adipocytes has been implicated in the control of adiponectin secretion and lipoprotein lipase activity. In the liver, CB_1 stimulation increases hepatic lipogenesis through activation of fatty acid biosynthetic pathway. This pathway is also expressed in hypothalamus, and is considered to be involved in regulation of appetite (76). Both CB_1 and CB_2 receptors are coupled to similar transduction systems.

Several lines of evidence have firmly established the role of endocannabinoid signaling in feeding behaviour, obesity and lipogenesis (77). Experimental studies have provided support for the role of endocannabinoids in obesity. In genetic rodent models of obesity such as *ob/ob* and *db/db* mice and Zucker rats, elevated levels of endocannabinoids were demonstrated in

the hypothalamus. The levels were normal in other regions of brain, and in nonobese control mice (78). Additional evidence indicates that endocannabinoids are involved in the neural circuitry of arcuate nucleus through which leptin regulates feeding behaviour. Leptin administration in *ob/ob* mice leads to a decrease in feeding alongwith a concomitant reduction of anandamide expression in the hypothalamus. In summary, in response to an increase in body weight, when leptin levels increase, anorexigenic neuropeptides such as NPY are upregulated, and orexigenic endocannabinoids levels are decreased, resulting in a reduction in CB_1 receptor activation, and a subsequent decrease in food intake.

Daniela Cota et al. (75) in a series of experiments used male mice deficient for CB_1 ($CB_1^{-/-}$) and male wild type (WT) littermates ($CB_1^{+/+}$). *In situ* hybridization in WT $CB_1^{+/+}$ mice confirmed that CB_1 transcripts are co-localised with mRNAs of several hypothalamic neuropeptides involved in control of food intake. In particular, co-localisation of CB_1 and prepro-orexin mRNA, and MCH mRNA was demonstrated in lateral hypothalamic area. Interestingly, CB_1 is neither expressed in NPY neurons in the arcuate nucleus, nor do $CB_1^{-/-}$ mice show altered levels of NPY mRNA expression.

The lack of CB_1 in mice with a disrupted CB_1 gene causes hypophagia and leanness. No significant difference between WT and $CB_1^{-/-}$ mice was detected regarding locomotor activity, body temperature or energy expenditure. CB_1 mRNA was found in epidydeinal fat pads from $CB_1^{+/+}$, but not from $CB_1^{-/-}$ mice. Altered expression of hypothalamic neuropeptides in $CB_1^{-/-}$ mice along with altered peripheral lipogenesis supports a role of the endogenous cannabinoid system in the central regulation of food intake and peripheral lipogenesis.

Horvath (79) has provided a schematic illustration showing the relationship between hypothalamic peptidergic circuits that express CB_1 receptors. It is of interest to observe the localization of these receptors in the MCH and orexin containing neurons in the lateral hypothalamus. CB_1 receptors are produced in the cytosol of these cells and are transported to axon terminals. Here, the receptors, upon activation by endocannabinoids or other agonists, are thought to affect the release of neuromodulators (MCH, ORX, CART, corticotrophin-releasing factor) to the synaptic cleft, thus modulating putative inhibitory (-) and stimulatory (+) influences on food intake through the various elements of the peptidergic system.

Neuropharmacological approaches to management of obesity and diabetes mellitus :

Before concluding the present narrative, the arclight must shift and be finally focussed on my own abiding commitment i.e. diabetes. In 1976, I had hypothesized that : *'a clear delineation of possible alterations in the normal physiological control mechanisms involved in the entero-hypothalamo-insular axis is likely to provide better insights in the diagnosis as well as management of diabetes mellitus.* ***Future development of specific neuropharmacological agents, modifying entero-hypothalamo-insular-axis, remains***

a distinct therapeutic possibility in the management of diabetes mellitus (8) '. Three decades later, with the discovery of CB_1 antagonists and incretins the time has come for the fulfillment of this intuitive prophecy. The subject has been extensively reviewed recently (80) and the following narrative is cited from this publication.

Clinical Trials with CB_1 receptor antagonists :

Following the mandatory animal studies, investigations were initiated in 2002 in the human subjects administered Rimonabant (SR 141716), the first specific CB_1 receptor antagonist. At present, multinational, multicentred, well designed clinical trials have provided sufficient data based on either a completed 1-year or 2-year study, or in an ongoing 2-yr. study. The following clinical trials are specifically designed to study the metabolic end-points (Table 1) :

Table 1. Rimonabant in Obesity (RIO) : Clinical Trials

Clinical Trial	Duration and Design	No. of Subjects	Dose of RIO	Efficacy
RIO – North America	2-yr. study: double blind, placebo controlled	3040 subjects : overweight or obese (Diabetics excluded)	5 mg. or 20 mg., oral, daily	62.5% treated with 20 mg. over 2 yrs. lost >5% body wt. HDL ↑ 24.5%, TG ↓ 9.9%, HOMA-IR ↓
RIO – Europe	2-yr. study: double blind, placebo controlled	1057 subjects : overweight or obese (Diabetics excluded)	5 mg. or 20 mg., oral, daily	* At 1-yr., 39% lost >10% body wt. with 20 mg. dose. Also waist circumference reduced by 3.4 inches. HDL ↑ 27%, TG ↓ 10.6%
RIO – Diabetes	1-yr. study: double blind, placebo controlled	1042 subjects with Type 2 diabetes; mean BMI ~34. 2/3 of subjects on metformin and 1/3 on sulphonylureas.	5 mg. or 20 mg., oral, daily	** On 20 mg. dose, HbA1c ↓ 0.6% in all patients and to < 6.5% in 43%. HDL ↑ 15.4%, TG ↓ 9.1%, average wt. loss of 11.7 lbs.
RIO – Lipids	2-yr. study: double blind, placebo controlled	1036 subjects : Obese,(BMI 27-40) with dyslipidemia.	5 mg. or 20 mg., oral, daily	***44.3% lost >10% body wt. with 20 mg. Waist circumference reduced by average 3.5 inches.

(Data tabulated by J.S. Bajaj)

HDL, high-density lipoprotein; TG, triglyceride; HbA_1c, haemoglobin A_1c; HOMA-IR, homeostasis model assessment-insulin resistance.

** Data published after completing 1-yr (study ongoing).*

*** Data presented at American Diabetes Association meeting, June, 2005.*

**** Data presented at American College of Cardiology meeting, March, 2005.*

Table 1 summarizes the available information based on the clinical trials with Rimonabant where the focus is on metabolic end points such as body weight, blood lipids, and glycemia. In general, 20 mg. dose is more effective with regard to all metabolic parameters. As the only study so far published in a peer reviewed medical journal (Lancet) is based on 1-yr. data in the RIO-Europe trial, the following critical review with regard to efficacy and adverse effects is based on this study (81). Basal metabolic rate was estimated with the Harris Benedict formula, and 600 Kcal were subtracted by a dietician to calculate a recommended daily energy intake for each subject. Total weight loss in 1-yr. ranged from 5 Kg. in the placebo group to more than 10 Kg. in subjects on daily dose of 20 mg. rimonabant. Waist circumference reduced by 3.4 cm. in 20 mg. dose group. HDL-cholesterol increased by 22.3% (20 mg. dose group), 16.2% (5 mg. dose group) and 13.4% (placebo group). Triglyceride decrease was observed only in 20 mg. dose group; in contrast, in the placebo and 5 mg. dose groups, an increase was observed. Finally, with 20 mg. dose, there was a significant reduction in fasting plasma glucose. A similar pattern was observed for insulin concentration as well as for HOMA-IR. No significant change in fasting glucose, insulin or HOMA-IR was observed in either the placebo, or the 5 mg. dose groups.

An analysis of all the adverse events occurring in at least 5% of subjects in any group showed that the most frequently reported adverse events with rimonabant were : nausea, dizziness, arthralgia and diarrhea. These events were generally mild to moderate and presented mostly during the first month of treatment. The only adverse event which occurred more frequently in the 20 mg. groups (compared to placebo and 5 mg. group) was pertaining to mood disorders.

The authors concluded that treatment with rimonabant was associated with clinically significant weight loss and reduction in waist circumference, with additional improvements in HDL-cholesterol, and a reduction in insulin resistance. The drug showed a favourable safety profile.

In addition to the four clinical trials with metabolic endpoints (Table1), there are additional studies with rimonabant and tobacco use (STRATUS) where the role of the drug in facilitating smoking cessation, long-term abstinence, and prevention of weight gain upon smoking cessation, is being investigated. More than 6500 subjects have been enrolled in the STRATUS trials.

Epilogue :

After more than 5 decades, the journey initiated by the original observations of Dr. Anand, seems to be heading for its final destination which now appears to be well within sight. The final conclusion that can be unequivocally pronounced is that although there is no 'conventional' feeding *centre* in the LH, there are certainly well characterized neurons located in this area which coexpress orexigenic neuropeptides along with CB_1 receptors and are involved in the regulation of feeding behaviour within the metabolic framework of

maintaining energy balance. There is also substantial evidence for the existence of entero-hypothalamo-insular axis. The newer therapeutic interventions in the management of diabetes mellitus such as Incretins and CB_1 antagonists directly arise from the basic research in this area.

To many, interdisciplinary research is a most recent concept. It is not. Aristotle (384-322 BC), himself a doctor's son, went to Athens to study with Plato, and was among the early pioneers to use animal dissection for learning the interrelationship between the structure and function of various organs. With a prophetic vision, Aristotle stated :

'The natural scientist has to investigate also the basic causes of health and disease, which cannot occur in non-vital things. That is the reason why most of the natural scientists finally turn towards medical research, while the more advanced and far sighted physicians will utilize the principles of natural sciences.'

The present scientific odyssey has been most exciting and amply rewarding.

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It was my good fortune to be associated with a group of scientists of international repute constituted by Dr. B.K. Anand, who was trained as a physician but turned into a physiologist by serendipity, late Dr. Baldev Singh, the doyen of neurologists but deeply involved in basic research in neurophysiology, and Prof. G.S. Chhina, an outstanding physiologist with major interest in applied clinical research including yoga. I pay my homage to late Dr. Baldev Singh and thank Prof. Chhina, Dr. Mohan Kumar and a large number of students and colleagues with whom I worked over the years. Indeed, this was the strength of the AIIMS in its formative years that facilitated close interaction and collaborative research between clinical and basic scientists. The present faculty should be well rewarded if they continue to follow those traditions. I also thank my wife, Avninder who unhesitatingly and most enthusiastically has always supported me to continue my academic pursuits for more than 40 years.

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