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Entero-Hypothalamo-Insular Axis Revisited

JS Bajaj

M.D., F.R.C.P.(Ed)., F.R.CP. (Lond.); F.A.M.S.
D Sc. (h.c. MGR Med. Univ.); D.Sc. (h.c. GND Univ.)
Hon. D.Sc. (Madras); D.M. (h.c. Karolinska)
Hon. D.Sc. (Banaras Hindu University)
D.Sc. (h.c. Univ. Health Sc; Andhra)

PREAMBLE

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. Based on our collaborative studies, Bajaj et al in 1975 proposed the existence of an Entero-hypothalamo-insular axis[4]. Subsequently Bajaj (1976)[5] summarised the evidence for its metabolic role delineating neuroendocrine mechanisms involved in the regulation of energy balance. In this publication, it was specifically observed: *'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'*. The present review provides a critique of information generated during the last two decades and assess the validity and authenticity of the foregoing statement in the context of current state of knowledge.

Thus, two physiological control systems seem to operate constantly and efficiently, essentially by building sufficient carbohydrate and triglyceride reserves and by ensuring their regular replenishment and mobilisation. A complex interplay of nervous, hormonal and metabolic pathways is involved in

The review is dedicated to the memory of Prof. Baldev Singh who along with Prof. G.S. Chhina closely collaborated with the author in several experimental studies which formed the basis of the concept of entero-hypothalamo-insular axis.

this process. Our understanding of actions of insulin and glucagon, of the metabolic processes such as gluconeogenesis and glycogenolysis on the one hand and of lipogenesis and lipolysis on the other, and of the neuroregulation of secretion and action of other hormones regulating and controlling intermediary metabolism, has been facilitated by rapid developments in the fields of molecular biology and immunology. A clearer picture has therefore emerged since the time our group (G.S. Chhina, late Dr. Baldev Singh and J.S. Bajaj) published our first review of the subject in 1972[1], and followed it up with a series of publications based on additional experimental studies (Bajaj et al 1974 a, b[2,3]; Bajaj et al, 1975[4]). The present review is an attempt to establish the continuity of our early work underlying the conceptual framework of entero-hypothalamo-insular axis, with the subsequent developments in this field, finally providing an insight into the state of the art at the turn of the millennium.

HISTORICAL PERSPECTIVE

Neuroregulation of Glucose homeostasis

Prior to our studies referred to above, there seemed to be a general consensus among the investigators regarding the following key aspect of brain glucose metabolism, with the tacit assumption that:

(i) insulin was not required for utilisation of glucose by the central nervous system;

(ii) insulin did not, and indeed was not capable of, crossing blood-brain barrier (BBB), and thus produced no metabolic effects in the brain.

In contrast, entero-hypothalamo insular axis (EHI) proposed by us was a radical departure from the generally held view, and operated on the premises that:

(i) insulin affects glucose utilisation of neurones in the ventromedial (VMH) and lateral hypothalamus (LHA), thus modulating neuronal activity in these areas;

(ii) electrical stimulation of LH in the conscious rhesus monkey stimulated insulin secretion from pancreatic beta cells; and

(iii) similar electrical stimulation of VMH resulted in a decrease in pancreatic insulin secretion.

These premises, along with the demonstration of evoked responses (ER) from the VMH and LHA following stimulation of mesenteric nerve and the changes produced in the ER following acute administration of glucose and insulin, provided the essential scaffolding for our proposed hypothesis.

Entero-hypothalamo-insular Axis

The entero-hypothalamic afferents of the proposed axis were demonstrated through the evoked responses (ER) elicited from the defined regions of hypothalamus as a result of electrically stimulating mesenteric nerve in anesthetised cat, with stainless steel bipolar electrodes implanted

sterotaxically in the VMH and LHA. Mesentric nerve was stimulated by employing positive square wave pulses of 0.05 - 0.2 msec duration, and an amplitude of 5-15V (Mohankumar 1971[6]; Chhina and Bajaj, 1972[1]). From the VMH evoked responses with a prominent negative phase were obtained. In contrast, ER from the LHA almost always showed the first deflection of positive polarity followed by a negative phase. The reciprocal relationship between the state of excitatory neuronal activity in the VMH and the inhibitory activity in LHA was confirmed by the consistent observation that the negative phase of ER in the VMH corresponded with the positive phase of the evoked response from the LHA.

Intravenous glucose administration produced a potentiation of the ER from VMH; this effect was more pronounced on the negative phase and was demonstrable within one minute of glucose adminitration. In contrast, a decreased (inhibitory) response in the amplitude of both the negative and positive phases was observed from the LHA concurrently with the increase (excitatory) in the negative phase ER from VMH. Intravenous administration of insulin resulted in a sudden transitory increase in the amplitude of ER from the VMH; this lasted for about 5 minutes and was followed by a decrease in ER amplitude. The ER from LHA immediately following insulin administration showed a decrease in amplitude of both phases, with complete recovery of the response in about 5 minutes, followed by a slight potentiation of negative phase after a further lapse of 5 minutes (Fig. 1).

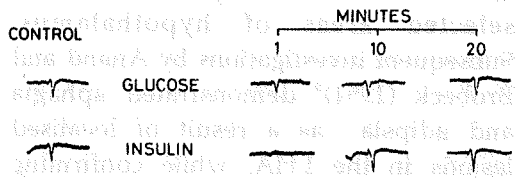

Figure 1. Effects of glucose and insulin on ERs from lateral hypothalamus on stmulation of mesenteric nerves. Glucose produced initial drop in amplitude which gradually recovered and then even increased (not shown in this figure). Insulin caused an initial short-lasting inhibition followed by an increase in emplitude (From: Chhina and Bajaj, 1972; Bajaj et al., 1975).

While these experiments provided evidence for the existence of some of the intestinal afferent pathways to hypothalamus, the afferent (effector) part of the EHI axis were elucidated by a series of experiments in normal and streptozotocin-induced diabetic rhesus monkeys as reviewed in the following paragraphs.

Hypothalamus: Then and Now

Chronologically, the credit for demonstrating the role of hypothalamic mechanisms in the regulation of energy balance must be given to Heterington and Ranson who in 1940, produced obesity in the rat by lesions confined to VMH. Subsequently, Brobeck et al (1943)[7] showed that the hypothalamic obesity was due to hyperphagia. The functional role of hypothalamus through autonomic nervous system received major attention culminating in the award of Nobel prize

to Walter Rudolph Hess in 1949 for his poineering studies on hypothalamus and autonomic function. Such a recongnition provided further impetus to those involved in the study of leisons in selected areas of hypothalamus. Subsequent investigations by Anand and Brobeck (1951)[8] demonstrated aphagia and adipsia as a result of localised lesions in the LHA, while confirming hyperphagia and obesity as a result of lesions restricted to the medial portion of the hypothalamus. They proposed the terms 'feeding' and 'satiety' centres to describe defined areas in the lateral and ventromedial hypothalamus, respectively.

Bernardis and Bellinger in a recent review (1993)[9] concluded that several of the changes originally ascribed to lesions in LHA might be due to a reduction in food intake, while a number of metabolic alterations were possibly due to a 'true' lesion effect, involving profound changes in glucose metabolism such as glycolysis, glycogenesis and gluconeogenesis. It is argued that the rats with LHA lesions regulate their body weight 'set' point in a primary manner, and not because of the destruction of a feeding centre. In the early stages of the syndrome, catabolism and running activity were enhanced, and so was the activity of the sympathetic nervous system as shown by increased excretion of norepinephrime which tends to normalize after about 4 weeks. Such observations provided the requisite interface between the feeding behaviour and the autonomic activity, both regulated by the hypothalamus.

Additional data on VMH lesions provided a striking complementarity and a cohesive conceptual matrix. In addition to hyperphagia the VMH-lesioned rats show hyperinsulinemia. Furthermore, in the first phase following VMH lesion, rats are hypersensitive to insulin with exaggerated response to small doses. In contrast, as obesity becomes manifest, VMH-lesioned rats become insulin resistant with a decrease in sensitivity and responsiveness of tissues such as liver and muscle to the administration of hormone (Penicaud et al, 1986)[10].

There is progressive development of insulin resistance in the muscle after the lesion of the VMH. Six weeks after the lesion, the muscle of the lesioned animals utilised less glucose than those of controls. Simultaneously, there was a transient insulin hypersensitivity in the white adipose tissue, wherein glucose utilisation was increased more than two-fold after one week but returned to normal in six weeks. This, together with hypersecretion of insulin, possibly contributed to the increase in body fat mass by redirecting glucose towards the adipose tissue (Penicaud et al, 1989)[11].

In our studies, different hypothalamic areas, namely the VMH, LHA, preoptic, posterior hypothalamus and mamillary body were stimulated through stereotaxically implanted electrodes in conscious male rhesus monkeys. A significant decrease was observed in blood glucose following the VMH and the preoptic area stimulation. An opposite response was obtained from LHA and posterior hypothalamus. There was a

generalised significant increase produced in serum free fatty acids, but triglycerides and cholesterol remained largely unaffected (Bajaj et al, 1974a)[2].

A 4-to 6-fold increase in circulating immunoreactive insulin was observed following the LHA stimulation (Fig. 2). An opposite response was obtained from the VMH (Fig. 3). Thus, insulinogenic and insulinoprival responses were obtained from feeding and satiety centres, suggesting a significant role of these areas

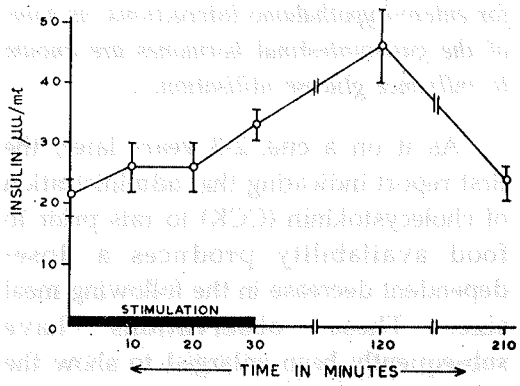

Figure 2. Effect of electrical stimulation of lateral hypothalamus on serum insulin.

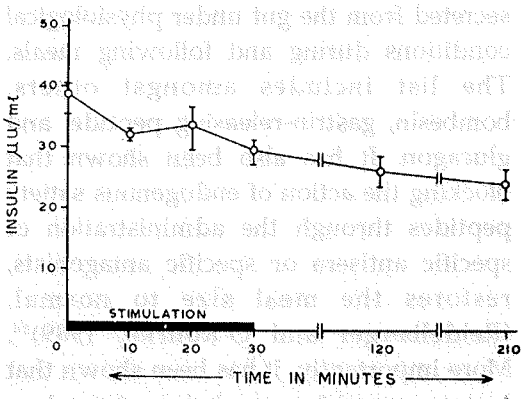

Figure 3. Effect of electrical stimulation of ventromedial hypothalamus on serum insulin.

in insulin regulation (Chhina and Bajaj, 1972[1]; Bajaj et al, 1974 a)[2]. The growth hormone and cortisol release were facilitated by the stimulation of the LHA and the VMH (Bajaj et al, 1974 a)[2]. The endocrinal responses from preoptic area resembled that of the VMH. The posterior hypothalamus and mamillary body showed trends which were common to both the LHA and VMH (Garg et al, 1983)[12].

Electrical stimulation of the VMH, in streptozotocin-induced diabetic, conscious male rhesus monkeys, significantly increased growth hormone, and decreased blood glucose. Serum insulin, free fatty acids, triglycerides and cortisol were largely unaffected, in contrast to the normal control animals where the insulin level showed a significant decrease. Cortisol and free fatty acids increased significantly by the VMH stimulation. None of the biochemical parameters showed any significant change at any time following the electrical stimulation of the parietal cortex. Thus the VMH stimulation did not alter the diabetic syndrome drastically. It also did not prevent the changes in metabolism seen after VMH stimulation in control animals (Garg et al, 1980)[13]

Insulin and Hypothalamus

Three years after the postulation of entero-hypothalamo-insular axis, the first supportive evidence for the role of insulin in CNS came from the studies of Jessie Roth's group (Havrankova et al 1978 a,b)[14,15] who demonstrated the presence

of insulin in the rat brain, with the concentrations in the hypothalamus being 2-3 fold higher than in other regions of brain excepting olfactory bulb[14,15]. Likewise, insulin receptors, with the largest population in the hypothalamus, were convincingly documented. Subsequently, expression of proinsulin messenger RNA by neurones (but not glial tissue) isolated from neonatal rabbit brain was documented (Schechter, 1988)[16]. A similar proinsulin mRNA expression was also demonstrated in neuronal cultures (Schechter et al 1990)[17]. Interestingly proinsulin mRNA expression was localised only to the neurones in the periventricular hypothalamus, an area suggested to have a role in feeding behaviour.

Thus, the earlier and traditional belief suggesting brain as an insulin-insensitive tissue, which was directly challenged and rebutted by the experimental evidence based on our collaborative studies (cited above), has now been substantially repudiated and our contention regarding metabolic effects of insulin on neurones in the hypothalamus, largely validated and accepted Indeed, insulin is recognised as an afferent CNS signal which regulates normal energy balance; although the contention whether it is synthesised *de novo* in brain, or is largely transported from plasma through blood brain barrier, remains a matter of ongoing debate.

Satiety hormones and peptides

While discussing the role of gastrointestinal hormones as possible modulators of neuronal signalling and as regulators of gastrointestinal blood flow, motility and absorption (Bajaj, 1976)[5], a reference was made to our observation regarding an increase in the circulating levels of gut GLI (Glucagon-Like-Immunoreactivity) following VMH stimulation in conscious restrained monkeys. *It was further observed that: '... no other studies are available regarding the alterations in gastrointestinal hormones following ablation or stimulation of VMH and LHA. It is likely that when the results of such studies are made available in the future, these may provide further evidence for entero-hypothalamo interactions, as some of the gastrointestinal hormones are known to influence glucose utilisation....'.*

As if on a cue, 2-3 years later, the first report indicating that administration of cholecystokinin (CCK) to rats prior to food availability produces a dose-dependent decrease in the following meal size. These observations have subsequently been enlarged to show the effect on meal size of a large number of gut peptides following exogenous administration. These peptides are secreted from the gut under physiological conditions during and following meals. The list includes amongst others, bombesin, gastrin-releasing peptide, and glucagon. It has also been shown that blocking the action of endogenous satiety peptides through the administration of specific antisera or specific antagonists, restores the meal size to normal. (Reidelberger and O'Rourke, 1989)[18]. More importantly, it has been shown that satiety peptides modulate impulses through vagal afferents, in addition to

their effect through receptors in the brain. Such afferent neuronal information passes through the brainstem to hypothalamus where it is processed and integrated with diverse neuroendocrinal-metabolic cues.

Long-term regulation of energy balance

While the role of EHI axis during the last quarter of century since the term was first coined, has been further clarified, expounded and validated, there is no doubt that it largely operates as a short-term control mechanism. Likewise, even though the satiety peptides produce a demonstrable decrease in meal size when administered immediately preceding a meal, their repeated administration does not alter body weight. This is in direct contrast to the observation of aphagia and weight loss which follows the lesions of LHA in the rat. Additional cues, through alternate neurohormonal mechanisms, must therefore operate, aimed at maintaining constancy of body weight on a long-term basis.

It is in this context that the information during the last five years has been truly phenomenal. Essentially, it deals with two interrelated operational systems:

(i) Leptin-insulin system and

(ii) Agouti-melanocortin system

There is evidence to suggest a cross-talk between these two systems.

(i) Leptin-insulin system:

Ob and db genes and their products serve as ligand and receptors, respectively, for a central pathway controlling and regulating satiety state and metabolic rate.

The recessive obesity (ob) single gene mutation in mouse produces marked obesity and results in a type of diabetes resembling human NIDDM (Friedman and Leibel, 1992)[19]. The mouse ob gene was subsequently isolated by positional cloning (Zhang et al, 1994)[20].

These ob/ob mice, in addition to being severely hyperphagic, also have a low body temperature thus combining increased energy intake due to excess appetite with reduced energy expenditure, leading to explosive weight gain. Additional features, such as high circulating levels of glucose, insulin and cortisol, tend to indicate a wider role of leptin in mammalian physiology. Furthermore, these animals are infertile suggesting involvement of leptin in hypothalamic pituitary-gonadal axis (Cameron, 1996)[21]. All of these features are reversed by administering mice leptin, the product of ob/ob gene (Campfield, 1995)[22]. Not only is the fertility restored in ob/ob mice following leptin administration, such a treatment also accelerates the outset of reproductive function in normal rodents (Chehab et al, 1997)[23]. Thus plasma leptin may act as a signal of nutritional state, laying down the hierarchial priority of self-survival (feeding behaviour) over species survival (reproductive behaviour).

Fasting and exercise decrease ob gene expression while feeding increases expression in adipose tissue ob mRNA.

Interestingly, these changes parallel insulin levels in circulation (Becker et al, 1995)[24]. There is substantive evidence to indicate that the adipocyte-specific hormone leptin, the product of the ob gene, regulates mass of adipose tissue through effects on satiety and by regulation of energy expenditure. In contrast to the satiety peptides such as CCK, the repeated administration of leptin induces weight loss by decreasing food intake and/or increasing energy expenditure (Levine et al 1996)[25]. Leptin acts through a single transmembrane domain receptor gene which is located on chromosome 4 in the mouse. The long form of the leptin receptor is primarily expressed in hypothalamus, while the short forms of leptin receptor are expressed in other areas of the brain, as well as in several other tissues (Lee et al 1996)[26]. In situ hybridization with antisense riboprobe shows strong expression in ventromedial, paraventricular and arcuate nuclei of hypothalamus (Mercer et al 1996)[27]. In the mouse, homozygous mutation in gene encoding leptin receptor causes obesity, hyperphagia and reduced energy expenditure. A recent publication (Montague et al, 1997)[28] describes the association of early-onset obesity with a mutation in the leptin gene. As was only to be expected, a subsequent publication followed early this year, describing for the first time human obesity and pituitary dysfunction as a result of a mutation in the human leptin receptor gene (Clement et al 1998)[29]. The authors conclude: ' Our results indicate that a functional leptin receptor is required not only for the regulation of body weight but also for sexual maturation and for secretion of growth and thyrotropic hormones. Leptin is therefore a critical link between energy stores and hypothalamic pituitary functions in humans'.

Hormonal regulation of Leptin

Circulating leptin levels remain unchanged following food intake in human subjects, suggesting a lack of effect of postprandial hyperinsulinemia on circulating leptin (Considine et al 1996)[30]. Likewise combined glucose and tolbutamide challenge, which is a major stimulus for β-cells, did not produce any significant change in basal leptin levels in lean insulin-sensitive, lean insulin-resistant and obese insulin-resistant male subjects with normal glucose tolerance (Segal et al 1996)[31]. However, small but significant gender difference may be present as shown in the study of Kennedy et al (1997)[32] wherein a 20% rise in basal leptin was observed in the females subjected to hyperinsulinemic-euglycemic clamp while no effect was demonstrable in male subjects under similar experimental conditions.

In contrast to in vivo studies in the human, in vitro studies using differentiated human adipocytes, a stimulatory effect of insulin on leptin secretion was consistently demonstrated (Wabitsch et al 1996)[33]. The reasons for differential in vivo and in vitro effects, and their physiological significance if any, need further elucidation.

In summary, leptin has a dual regulatory function in human physiology,

affecting feeding and reproductive behaviour. During the period of weight maintenance, when energy intake equals energy expenditure, circulating leptin levels reflect total body fat mass. However, during a significant alteration in body weight, leptin levels serve as sensor of energy imbalance: increase in body fat-mass results in enhanced leptin secretion from peripheral adipocytes while even a short term fasting decreases leptin secretion, with circulating leptin levels declining to nearly 30% of initial basal levels. Unlike marked changes in serum leptin, CSF leptin is only moderately increased in obese subjects and the CSF leptin/serum leptin ratio decrease logarithmically with increasing body mass index.

Mechanism of Leptin Action

The regulatory pathways underlying central action of leptin have recently been reviewed and summarised (Flier and Maratos-Flier, 1998)[34]. At the onset of obesity, with increasing triglyceride storage in adipocytes, leptin secretion is enhanced, resulting in increase in both the peripheral and CSF circulating levels of leptin. In the CNS, leptin acts on receptors in the arcuate nucleus of the hypothalamus, and inhibits secretion of neuropeptide Y (NPY). NPY is a 36 aminoacid polypeptide synthesised by neurones in the CNS as well as in the peripheral nervous system. In the CNS, NPY synthesis is localised to neurones in the arcuate nucleus of hypothalamus and its subsequent release is through their axons in the paraventricular nucleus. The production of NPY (in the arcuate) and its secretion (in PVN) is affected by the state of energy balance. Increased levels of leptin, in response to increased adipocyte mass, suppress appetite and reduce food intake by inhibiting NPY synthesis and release, thereby constituting a major regulatory mechanism.

It is of considerable interest to note the role of NPY as a common regulatory pathway, shared both by leptin and insulin. Like leptin, insulin also is a peripheral adiposity signal to CNS. Such a contention is supported by a large dataset cumulated over the last few years: basal plasma insulin levels show marked increase in obesity as well as in those states characterised by a reduction in every expenditure; there is a dose-dependent entry of peripheral circulating insulin into the CNS; neurones express high concentration of insulin receptors in hypothalamic areas known to regulate feeding behaviour; and finally, the most significant effect of small doses of insulin following either central or peripheral administration (so long as hypoglycemia is avoided) is to produce a marked reduction of food intake and a state of negative energy balance resulting in weight loss. That such an effect is mediated through the VMH was shown by an increase in food intake following acute injection of insulin antibodies into the VMH of rats during dark portion of day/night cycle (to time it with the natural diurnal eating pattern in the rat). More recently, McGowan et al (1990)[35] have reported a more chronically increased food intake and rate of weight

gain following repeated chronic infusion of insulin antibodies into ventromedial hypothalamus in the rat.

There is now evidence to suggest that like leptin, insulin also mediates its effects through NPY. Inhibition of hypothalamic NPY gene expression by insulin has been reported (Schwartz et al, 1992)[36]. Complementary data, supporting insulin - NPY interaction in the hypothalamus has been provided in both the spontaneously diabetic (non-insulin dependent) BB and in streptozotocin - induced insulin-dependent animals rat (Abe et al, 1991)[37]. In the latter, with marked insulin deficiency, augumentation of hypothalamic expression of preproNPY mRNA was demonstrated (Sahu et al 1990)[38]. Both the NPY and its mRNA showed increased hypothalamic levels in insulin deficient animals, and both were normalised following systemic insulin therapy. In this connection, it may be of interest to recall our data regarding bipolar EEG recording in the normal and streptozotocin-induced monkey which showed a slower activity in the ventromedial hypothalmus, returning to normal after insulin administration (Bajaj, 1976)[5]. It is therefore justified to suggest that both the EEG changes in VMH and hyperphagia in the diabetic monkey, were possibly due to augumentation of NPY levels in the arcuate-paraventricular nuclei. The changes were reversed following systemically administered insulin therapy. Both insuln and leptin are secreted in increased amounts in obesity, and both act through central receptors involving NPY system.

(ii) Agouti-Melanocortin system

Recent studies based on cloning and characterization of a number of mouse obesity genes (Ob and db genes have been discussed in detail above), such as agouti, tub and fat have provided mutant mice which are suitable models of moderate, slow-onset mouse obesity, that may be closer to adult-onset obesity (and diabetes) in the human (Naggert et al 1995[39]; Noben-Trauth et al 1996)[40]. Agouti was the first amongst these obesity genes to have been cloned (Bultman et al, 1992)[41]. This gene is normally involved in coat colour regulation in mice. It encodes a 131-aminoacid molecule which exerts a paracrine action on melancocytes. Dominant mutations at the agouti gene result in mice, with predominantly yellow fur, characterised by progressive obesity, hyperinsulinemia, peripheral insulin resistance, impaired glucose tolerance, decreased thermogenesis and mild hyperphagia (Liebel et al, 1997[42]; Michaud et al, 1997)[43]. As agouti modulation of pigmentation is mediated through the fact that the agouti protein competitively inhibits αMSH binding to melanocyte melanocortin-1 receptor (MC1-R), studies were initiated to investigate whether a similar antagonism at other MC-receptors may form the basis of the metabolic syndrome in agouti mutant mice. In addition to MC1-R, there are four other members of the MC-R family: MC2-R is the ACTH receptor; MC3-R is found in the hypothalamus and limbic system; MC4-R is widely expressed in may regions of the brain, and MC5-R is expressed in a large number of body

tissues. As both MC3-R and MC4-R are expressed in the VMH, it was suggested that agouti antagonism at these sites may explain hyperphagia. Indeed, targetted disruption of the murine MC4-R (Husgar et al, 1997)[44] and the recent data from a family study in Quebec (Chagnon et al 1997)[45] tend to support such a hypothesis.

Several studies indicate that there is a significant cross-talk between the leptin and agouti-melanocortin signaling pathways. Leptin, while inhibiting secretion of NPY, increases the release of (MSH and at the same time decreases the secretion of agouti-related peptide (AGRP) which is an antagonist of αMSH at melanocyte melanocortin-4 (MC4) receptor and is expressed in several regions of the brain. It is the MC4 receptor which is 'feeding-inhibitory'. The net output from the feeding inhibitory MC4 signalling pathway located in the ventromedial hypothalamus may be determined by the ratio of agonist (αMSH) and the antagonist (AGRP) at the MC4 receptor neurone. Leptin alters the ratio in favour of αMSH/ AGRP by increasing αMSH, and reducing AGRP secretion, in the ventromedial hypothalamus thus suppressing food intake.

It is abundantly clear that for the first time, it is possible to visualize molecular basis of signalling of 'satiety' neurones, described by Anand and Brobeck nearly 50 years ago, and followed by our subsequent studies resulting in the concept of entero-hypothalamo-insular axis, and its role in energy balance on a long term basis.

OREXINS : The Feeding Molecule(s)

The mechanisms of molecular signalling in the ventromedial hypothalamus and of the agouti-leptin-insulin cross talk in and through the neurons and synapses, have been amplified and properly 'tuned' to optimise the noise: signal ratio. In contrast, the 'silence' of the lateral hypothalamus remained intriguing till early this year when modern tools of molecular biology were used to explore this hitherto silent area. The result is an exciting and most fascinating development.

Using the modern methods of molecular recognition, Sakurai et al (1998)[46] discovered two key hypothalamic neuropeptides, now named Orexin A and B (Gk. *orexis*, appetite) which are located in the 'feeding' centre of lateral hypothalamus. Perhaps, recognising that the original observations of Anand and Brobeck wherein ablative lesions produced in the rat lateral hypothalamus resulted in aphagia and weight loss were true and valid, Sakurai et al set upon the task to find neuropeptide(s), if any, underlying the behavioral and metabolic changes which result from such lesions. Expressed sequence tags (ESTs) available from public databases, with sequence homologies to known G protein-coupled cell-surface receptors (GPCRs) were used for screening with human brain cDNAs to obtain full length cDNAs and fifty stable transfectant cell lines were generated as a result. Each of these cell lines expressed a distinct orphan GPCR cDNA. Crude peptide extracts from rat

brain were tested on the transfected cells for GPCR agonist activity by measuring increase, if any, in cytoplasmic Ca^{++} level, a common response to G protein receptor activation.

A major active peptide fraction was isolated, purified to homogeneity in four steps by HPLC, sequenced, and its structure established by mass spectrometry. The resultant peptide, called Orexin A consists of 33 aminoacids (MW = 3562 Da). A second peak of activity was separated and named Orexin B (MW = 2937 Da). Orexin A cDNA was made by reverse transcriptase treatment of rat brain mRNA followed by PCR. The structure of prepro-orexin gene has been determined, and it has been located on chromosome 17q21. Both the 33 aminoacid Orexin A and 28-aminoacid Orexin B are encoded by a single mRNA transcript. Also identified were two Orexin receptors. Orexin A receptor (Ox1R) showed a 26% identity with NPY. While Orexin A was a specific high affinity agonist for Ox1R stimulation, a second receptor Ox2R with 64% identify to Ox1R and high affinity binding of Orexin B has also been sequenced. Ox2R also binds Orexin A.

To localise Orexin expression within the CNS, in situ hybridization and immunohistochemical analyses in the rat brain was performed. It showed the presence of prepro-orexin mRNA bilaterally and symmetrically in the lateral and posterior hypothalamic areas and the perifornical nucleus. It is noteworthy that no signal was detected in neurons of paraventricular, ventromedial or arcuate neclei, areas which are known to contain neuropeptides such as NPY associated with food consumptin, which are leptin sensitive. Impressed by the 'striking localisation' of Orexin containing neurons in the lateral hypothalamus and some of its adjacent areas, and none in the VMH-Arcuate-PVN area, Sakurai et al investigated the effect of Orexin administration through preimplanted indwelling catheters. Within one hour of intracerebroventricular administration of Orexin A bolus, food consumption was stimulated in a dose-dependent manner. Human Orexin-B administered similarly in the rat also augumented food intake significantly but the duration of its action was shorter. These actions of Orexin were similar to, albeit of lesser order of magnitude, than those observed following NPY administration.

Epilogue

In concluding our paper published in 1976 (Bajaj, 1976)[5], it was stated:

*'Obesity resulting from a functional disorder of the hypothalamus remains a remote possibility. A clear delineation of possible alterations in the normal physiological control mechanisms involved in the EHI axis is likely to produce better insights in the diagnosis as well as management of diabetes mellitus. The possible therapeutic effects of yoga may be mediated through alterations either at the level of sensory inputs (entero-hypothalamic) or at the level of central receptor mechanisms (Bajaj, 1976 a)[47]. **Future development of specific neuropharmacological agents, modifying EHI axis remains a distinct***

therapeutic possibility in the management of obesity and diabetes mellitus'.

It is gratifying to note that nearly a quarter century later, in concluding the discussions of their paper (Sakurai et al, 1998)[46] amplify the above views, using their own work and citing other observations, eventually suggesting similar, if not identical, future possibilities. The concluding paragraphs of their discussion are reproduced below:

'Recent studies continue to reveal the molecular basis for the role of periventricular/medial hypothalamic regions in energy homeostasis, e.g., ventromedial nucieus, arcuate necleus, and paraventricular nucleus. Neurons containing neuropeptides such as NPY (Bing et al, 1996)[48], melanocortins (Jacobowitz and O'Donohue, 1978)[49], glucagon-like peptide-1 (Shughrue et al, 1996)[50], and galanin (Warembourg and Jolivet, 1993)[51], as well as the leptin and melanocortin-4 receptors (Mountjoy et al, 1994[52]; Tartaglia et al, 1995)[53] are abundant in one or more of these periventricular/medial hypothalamic regions. In contrast, few neuropeptides have been described to be produced chiefly in the lateral hypothalamic regions. Other than the orexins, we are aware of only one distinct neurotransmitter that is specifically produced in the lateral hypothalamus: melanin concentrating hormone (MCH) has been localised in the zona incerta and the lateral hypothalamic area (Bittencourt et al,. 1992)[54]. Intriguingly, MCH was recently reported to stimulate food intake upon central administration. Moreover, MCH mRNA is up-regulated in ob/ob mice and by fasting in wild-type mice (Qu et al, 1996)[55]. It will be important to investigate further the possible interplay of orexin with this and other positive (e.g., Agouti-related protein, NPY, galanin, and opioids) and negative (e.g., leptin, melanocortins, corticotropin-releasing factor, glucagon-like peptide-1, and cholecystokinin) regulators of energy balance both within and outside the central nervous system (Arase et al, 1988[56]; Rosenbaum et al, 1997)[57]

A decline of blood glucose levels can signal the initiation of food intake (Oomura, 1980)[58]. The lateral hypothalamic area contains glucose-sensitive neurons that are activated by glucopenia and thus implicated in the positive short-term regulation of feeding and energy expenditure. It is tempting to speculate that all or some of the orexin-containing neurons may be glucose-sensitive, or that they may receive stimulatory projections from glucose-sensitive neurons. Future experiments will also determine whether orexins have additional actions relevant to nutritional homeostasis, such as effects on the regulation of systemic energy expenditure, secretion of metabolic hormones such as insulin, and ultimately, the regulation of body weight.

The present discovery of orexins and their receptors may provide a novel molecular basis for the role of the lateral hypothalamic areas in the regulation of feeding behaviour. It is clear, however,

that the definitive assignment of physiological roles for orexins requires further pharmacological as well as molecular genetic investigations. ***Nevertheless, pharmacological intervention directed at the orexin receptors may prove to be an attrative avenue toward the discovery of novel therapeutics for diseases involving disregulation of energy homeostasis, such as obesity and diabetes mellitus.***

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It is immaterial that Sakurai et al (1998)[46] do not cite Bajaj (1976)[5]. What is important in science is the validation of original observations and the postulated hypotheses. Some of us including B.K. Anand, G.S. Chhina and myself are fortunate to see this happen in our own life time. Unfortunately, Sakurai's paper was published in the February 20, 1998 issue of the journal, Cell. Dr. Baldev Singh died on February 2, 1998 without knowing the work on Orexins !

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