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Insulin and Insulin Receptors in Rodent Brain

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Summary. While insulin effects on the central nervous system (CNS) mediated through hypoglycaemia are well known, direct insulin effects on the CNS remain controversial. Recently, we found insulin receptors in all areas of the rat brain, with highest concentrations in the olfactory bulb, cerebral cortex and hypothalamus; all areas involved in feeding. Insulin receptors in brain were, by multiple criteria, similar to insulin receptors on classical target tissues for insulin, such as liver and fat. Insulin itself has been identified in the rat brain at concentrations on average ten times higher than in plasma. Highest concentrations were found in the olfactory bulb and hypothalamus. Brain insulin was indistinguishable from purified insulin by its behaviour in the radioimmunoassay, radioreceptor assay, bioassay and gel chromatography. In two experimental models representing extremes of plasma insulin concentrations (obese hyperinsulinaemic mice and diabetic insulinopenic rats) there were no significant changes in the concentration of insulin receptors in brain while liver receptors were modified in the expected way. This may reflect the protective influence of the blood-brain barrier or some special quality of brain insulin receptors. Insulin concentrations in brain were also unchanged in both models, which is probably indicative of the local synthesis of insulin. The role of insulin in the CNS is unknown. Besides well known metabolic actions of insulin, new roles can be postulated such as neurotransmission, neuromodulation and paracrine signalling.

Key words: Central nervous system, CNS peptides, insulin receptors, insulin, experimental diabetes

Insulin is a potent regulator of many aspects of metabolism in almost all mammalian tissues [1]. The central nervous system (CNS) is currently considered by most to be independent of insulin action, at least as far as glucose metabolism is concerned. However, significant effects are observed when the CNS is exposed to insulin. After the administration of insulin into the carotid artery [2], the cisterna magna [3] or into the ventromedial hypothalamus [4], peripheral hypoglycaemia is observed within minutes. The effect is probably mediated through the parasympathetic nervous system, since vagotomy greatly diminishes the hypoglycaemic response to the centrally administered insulin [5]. Whether similar mechanisms are operative under physiological conditions is unclear, but there is no doubt that insulin can act on the CNS under some circumstances. Since it is believed that most, if not all, insulin effects are mediated through its receptor localized on the outer surface of the cell, one would expect to find insulin receptors in the CNS.

Insulin can reach the cerebrospinal fluid (CSF) and then, presumably, the brain tissue after its administration into the peripheral circulation [6,7]. The equilibrium between the periphery and the CNS is reached only after several hours and just a small fraction of the injected insulin can be found in the CSF. However, so called non-barrier regions [8] of the CNS (areas devoid of tight capillary junctions which elsewhere constitute the blood-brain barrier) are directly exposed to the circulating insulin. One important example of such a non-barrier region is the infudibular hypothalamus. Areas with an efficient blood-brain barrier probably cannot be reached by circulating insulin quickly enough for it to exercise an acute effect. If the brain had its own "stores" of insulin acquired either by local synthesis or by concentration from peripheral circulation and/or CSF, it would be relatively independent of peripheral insulin. Local release and response could be taking place. Recently, many peptides have been isolated from both the gastrointestinal tract and the CNS [9] and the list is continuously expanding [10].

We will review our experimental data leading to identification of insulin receptors and insulin in the CNS of rodents. Data suggesting the local synthesis of insulin in the CNS will also be presented.

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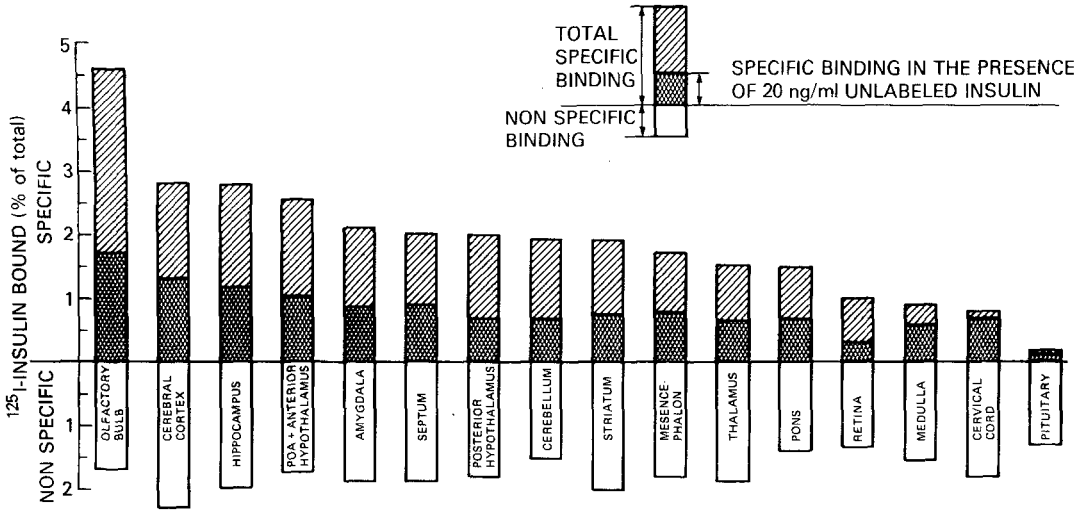

Fig. 1. *Regional distribution of the insulin receptor in the CNS of the rat.* Indicated regions were macroscopically dissected, homogenized in 1 mmol/l $NaHCO_3$ and centrifuged for 10 min at 600 g. The supernatants were centrifuged at 20000 g for 30 min and washed once. (All steps were performed at 4 °C). Resulting pellets were suspended in a Hepes containing buffer (11), exposed to porcine ^{125}I-insulin (130–180 μCi/μg) in the absence or presence of unlabelled insulin (20 ng/ml and 100 μg/ml) and incubated at 15 °C for 90 min. Tubes were then centrifuged in a Beckman microfuge and supernatants removed for the measurement of insulin degradation (estimated by precipitation with trichloroacetic acid, which ranged between 4 and 10%). The radioactivity in the pellet was counted in a γ-counter ("total binding"). The "non-specific binding" (in the presence of 100 μg/ml of unlabelled insulin), indicated by empty bars below the baseline, was substracted to obtain "specific binding", hatched bars above the baseline. The double hatched part represents the specific binding in the presence of 20 ng per ml of unlabelled insulin (indicating roughly the affinity of the receptor). The total, specific and non-specific binding was adjusted for each region to 500 μg of protein per ml. (From Havrankova et al. [11], used with permission)

Insulin Receptors in the CNS of Rodents

We identified insulin receptors on plasma membranes prepared from several discrete regions of rat brain [11]. As shown in Figure 1 the highest binding was observed in olfactory bulb and cerebral cortex. High levels of binding were also observed in hippocampus, preoptic area plus anterior hypothalamus, amygdala, septum and posterior hypothalamus, with somewhat lower binding in cerebellum, striatum, mesencephalon, thalamus and pons. The lowest binding was observed in the pituitary, cervical spinal cord, medulla and retina. The level of specific binding was equal to or greater than that observed with comparable membrane preparations from liver, a classical target tissue for insulin. The insulin receptor on the membranes of the cerebral cortex was most extensively studied. Its affinity, pH, time, and temperature dependence of binding were indistinguishable from those of other insulin receptors. Most importantly, insulin analogues, insulin-like peptides and unrelated peptides behaved in the way predicted from their in vitro bioactivity and their reactivity with insulin receptors of liver, muscle and fat.

Van Houten et al. recently identified specific binding sites in the circumventricular organs of the rat brain by radioautography [12]. Since labelled insulin was administered by the intracardiac route and animals were sacrificed five minutes later, it is not surprising that insulin receptors were demonstrated only in non-barrier regions. As pointed out previously, the blood-brain barrier is penetrated only slowly and incompletely by insulin [6,7].

It has been demonstrated both in vivo and in vitro that insulin is a major regulator of the concentration of insulin receptors [13]. The decrease in the number of insulin receptors in the presence of hyperinsulinaemia has been labelled "down regulation". The increase in the quantity of receptors induced by insulin deficiency is called "up regulation ". Is the same mechanism operative in the CNS?

We studied two experimental models representing extremes of plasma insulin levels. The genetically obese mouse (ob/ob) is hyperglycaemic and hyperinsulinemic. Rats treated with moderate doses of streptozotocin (65 mg/kg) become hyperglycaemic and insulinopenic. In both models the altered insulin levels were sustained for at least one month before the study. No changes were observed in the number or affinity of insulin receptors in five distinct areas of brain while the expected adjustments in the concentration of insulin receptors in the liver did occur (Figs. 2, 3) [14]. Given the heterogeneity of elements within the CNS, we cannot exclude that some changes may be occuring within smaller areas than those studied. This demonstrates that insulin receptors in the CNS are not regulated in response to circulating levels of insulin. This can be either due to the presence of the blood-brain barrier that prevents blood cells from registering changes in the level of

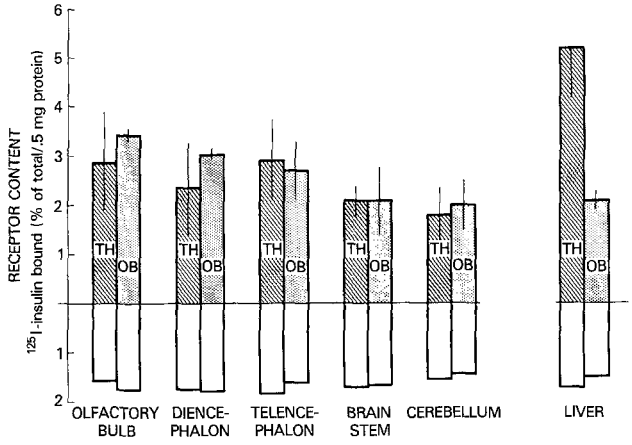

Fig. 2. *Insulin receptors in brain and liver of ob/ob mice (OB) and their thin littermates (TH).* 20000 g pellets were prepared from brain areas and liver of 6 to 8 weeks old mice and the radioreceptor assay was performed as described in the legend of Figure 1 except that Krebs-Ringer phosphate buffer was used instead of Hepes containing buffer. Bars above the baseline represent the specific binding; empty bars under the baseline represent the non-specific binding. The degradation of ^{125}I-insulin ranged between 4 and 8% for brain membranes and 15 to 30% for liver membranes. Degradation was similar with membranes from experimental and control animals. Binding was adjusted to protein content in each area. (From Havrankova et al. [14], used with permission)

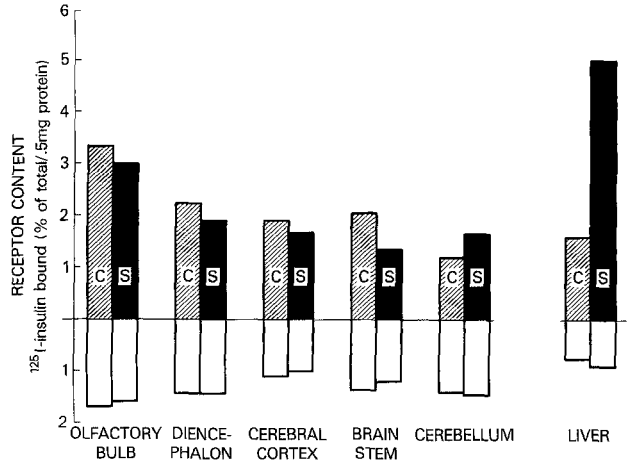

Fig. 3. *Insulin receptors in brain and liver of control (C) and streptozotocin-treated (S) rats.* 20000 g pellets were prepared from brain areas and liver of diabetic rats one month after induction of diabetes and control rats (injected with vehicle) and the radioreceptor assay performed as described in the legend of Figure 1. Membranes were prepared from tissues from three experimental and three control animals; each set of bars represents a mean of triplicates. The receptor content expressed as percent of specific binding of ^{125}I-insulin is shown above the baseline; the non-specific binding (in the presence of an excess of unlabelled insulin – 100 μg/ml) is depicted under the baseline by empty bars. (From Havrankova et al. [14], used with permission)

circulating insulin or to some special properties of brain cells that preclude adjustment of the concentration of receptors.

The 3T3-L1 fatty fibroblast is also rich in insulin receptors and does not "down regulate" when exposed to increased insulin concentrations [25].

Insulin in the CNS

Since only a fraction of circulating insulin reaches the CNS, it can be asked whether under physiological conditions the CNS is aware of changes in peripheral insulin levels. Why are there insulin receptors in the CNS? Is it Nature's mistake, putting receptors which cannot play any role on the cell surface or is it possible that the CNS supplies its own insulin?

We extracted rat brains using methods described for insulin extraction from pancreas [15]. By radioimmunoassay the concentration of insulin in the whole brain averaged 28 ng/g, which is about ten times higher than that of plasma [16]. Insulin is distributed unevenly within the CNS; highest concentrations are observed in the hypothalamus and olfactory bulb and the lowest in the cerebral cortex and brain stem. There is no apparent correlation between receptor concentration and insulin content. Brain insulin was indistinguishable from an insulin standard by radioimmunoassay, gel filtration, radioreceptor assay and bioassay. The bioactivity of brain insulin was neutralized by addition of antiinsulin antibody. Insulin was localized by an immunocytochemical method within nerve cell bodies in the olfactory bulb and frontal cortex [16].

Where does brain insulin originate? Either it is synthesized locally or it is concentrated from insulin present in blood and/or CSF during circulation through the CNS.

In trying to solve this problem we reasoned as follows: If brain insulin levels parallel changes in circulating insulin concentration, it is likely that brain insulin comes from the periphery and is concentrated locally. If, on the other hand, there is no change in brain insulin levels with hyper and hypoinsulinaemia, the hypothesis of local synthesis of insulin will be strengthened.

Obese hyperglycaemic mice have plasma insulin levels fifty times higher than their thin littermates. Insulin levels were reduced by 50% in streptozotocin-treated diabetic rats. In both models brain insulin concentrations were similar to their respective controls (Figs. 4, 5). If anything, there was an increase in insulin content of the hypoinsulinaemic rats, but this difference did not reach statistical significance. Thus, brain insulin content does not change in the presence of greatly altered peripheral insulin levels, suggesting local synthesis of insulin. The fact that proinsulin has not been found in brain extracts is not against the hypothesis of insulin being synthesized in the CNS for multiple reasons. Our antibody is more sensitive

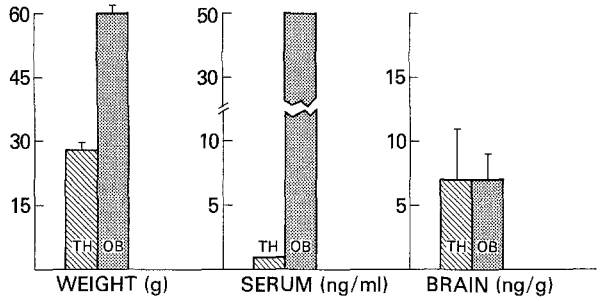

Fig. 4. *Insulin content of serum and brain of ob/ob mice (OB) and thin littermates (TH).* Eight obese mice and eight thin littermates were weighed; serum from each group was pooled and insulin concentration measured in the radioimmunoassay using guinea pig anti-porcine insulin serum (gift of Dr. A. Kagan), porcine ^{125}I-insulin (130–180 μCi/μg) and porcine insulin standard (Eli Lilly). Brains were homogenized by Polytron in 10 volumes of ice-cold acid-ethanol (HCl 0.2 N/ethanol 75%) and extracted overnight at 4 °C. Suspensions were then centrifuged and the supernatants concentrated by air evaporation at room temperature to about 1/10 to 1/20 of original volume. The concentrate was resuspended in about 5 volumes (relative to the original tissue weight) of $(NH_4)_2CO_3$ 0.05 M, 1 mg/ml bovine serum albumin, and neutralized with NH_4OH (concentrated). Samples were centrifuged and supernatants lyophylized and reconstituted in Veronal buffer for the insulin radioimmunoassay. To determine insulin losses during the extraction, a tracer amount of ^{125}I-insulin was added (10000 cpm ≃ 35 pg) at the beginning of the extraction procedure; radioactivity remaining at the end of the procedure was expressed as percentage of original counts ("recovery"). Reported insulin concentrations were corrected for recovery which varied between 30 and 60%. Eight brains from each group were extracted; the mean insulin content ±SEM is shown. (From Havrankova et al. [14], used with permission)

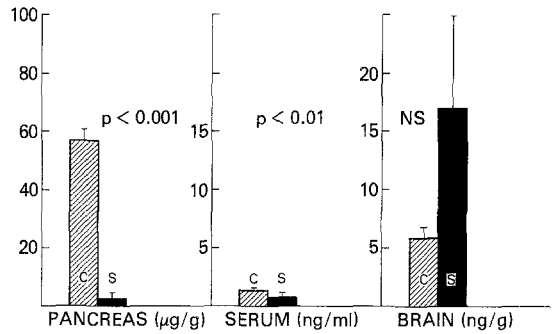

Fig. 5. *Insulin content of pancreas, serum and brain four weeks after induction of diabetes in eight streptozotocin treated rats (S) and eight controls (C).* Four weeks after the injection of streptozotocin or a vehicle, rats were sacrificed by decapitation, their serum collected and the pancreas and brain were dissected and extracted using the same procedure described in the legend of Figure 4. Insulin concentrations in serum and both tissues were measured in the same radioimmunoassay; the mean insulin concentrations ±SEM are shown. Values reported for insulin content of brain and pancreas were corrected for the recovery (Cf. legend to Figure 4). (From Havrankova et al. [14], used with permission)

to insulin than proinsulin. Even in pancreas, proinsulin constitutes only a few percent of the total insulin content [17]. Also, the CNS seems to be more effective generally than the gastrointestinal cells in the conversion of peptide precursors to final product [18]. While a mechanism for concentrating insulin from CSF or blood has not been completely excluded, it would be necessary to postulate a very finely tuned system which could maintain unchanging brain insulin levels in the face of great variation in peripheral insulin. Although the circumstancial evidence is in favour of local synthesis of insulin, more direct approaches will have to be used in order to provide conclusive evidence.

What is the role of insulin in the CNS? Insulin effects on glucose metabolism in the CNS are controversial; some authors found slight but definite effects [19], while others did not find any at all [20]. The unchanging brain insulin levels during profound alterations of plasma glucose and insulin probably points away from a significant role for insulin in brain glucose metabolism. It is well established that insulin is a growth promoting agent; thus it is possible for insulin to be playing that role in the CNS. It has been postulated by Porte and Woods that insulin is a long-term determinant of body weight [21]. This effect could be mediated through insulin receptors in the CNS, in particular in the hypothalamus. New roles for insulin can be envisioned such as paracrine signaling, neurotransmission or neuromodulation.

Interestingly, insulin has been found in peripheral nerves of the cat and this insulin can be released after stimulation of the nerve [22].

Other peptides, previously labelled "gastrointestinal", have been identified in the CNS, such as cholecystokinin, vasoactive intestinal polypeptide and gastrin. Other peptides have been first discovered within the CNS and then identified in the gut, such as neurotensin, thyrotropin-releasing hormone and somatostatin. It has been suggested that a common embryological origin of cells from neuroectoderm explains the common secretory product [23]. Although there is controversy concerning the neuroectodermal origin of GI cells, one can still retain the hypothesis of a common origin of cells in the brain and GI tract, and for that matter, of all cells in the body. They all originate from the same zygote, have the same genetic complement and a potential capability to synthesize all proteins of that species. Recently, we found insulin in significant concentrations in essentially all mammalian tissues [24]. Why is insulin so widely distributed in the organism? Is it simply a reflection of a lack of complete repression of the insulin gene, so called "gene leakage"? Are all peptide hormones (or all peptides) synthesized to some extent by all cells? Alternatively, is there any specific purpose for insulin in all tissues? We do not yet have these answers.

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Discussion after Havrankova's Presentation

Fernstrom: Is it possible to elevate brain insulin to the levels you measure by increasing plasma insulin?

Havrankova: At least two groups have shown that the transport of insulin into the brain, at least into the CSF, is very slow and incomplete (Nature (1967) 215: 1375; Am J Physiol (1977) 233: 331). We feel that so much insulin would have to be given that the animal would be in danger to die from hypoglycaemia before sufficient insulin could enter.

Steffens: Can insulin in the blood actually get into the brain tissue?

Havrankova: So far, it's only been measured in the CSF, but presumably some could get into the brain tissue via the CSF. The point, however, is that although some insulin may gain access to the brain in this way, there still must be considerable synthesis locally to account for the tenfold difference we observe.

Steffens: Is there a difference in the half-life of plasma and brain insulin? If so, it might also help to explain your findings.

Havrankova: It is unlikely that even a huge increase of the half-life could account for our observation that brain insulin doesn't change over one month after treatment with streptozotocin.

B. Jeanrenaud: Have you measured proinsulin in the CNS?

Havrankova: We have not been able to measure it; but even in the B-cells, proinsulin is only a small percentage of the total insulin and hard to measure, so we could easily have missed a few percent. Even if we were able to measure proinsulin in the brain it would not be conclusive since a transport system for insulin into brain might also be sensitive to circulating proinsulin.

Porte: Have you found C-peptide in the brain?

Havrankova: We haven't yet assayed for it.

B. Jeanrenaud: Have you tried to separate the cellular components where insulin binds?

Havrankova: Yes, we have separated various fractions and found that insulin binds specifically to a mitochondrial fraction and a synaptosomal fraction. It also binds to a glial fraction, but these data were not supported by Dr. van Houten.

Novin: Are the findings that insulin binds mainly to the microvessels contradictory to your findings?

Havrankova: Actually, I think they are complementary. Remember that Dr. van Houten has found receptors on the microvessels and the CVO's, but these are for blood-borne insulin. Our assay experiments are measuring a different pool of insulin, one which may be independent of that system.

Novin: Why do you think there is so much insulin in the olfactory bulb?

Havrankova: I really have no idea and the finding was unexpected. However, we have since learned that the olfactory bulb is very active metabolically in that it burns considerable glucose. Further, when the rat smells odours, the rate of glucose metabolism in the olfactory bulb increases (Brain Res (1975) 98: 596). There might therefore be a pathway involved in food intake since the olfactory bulb connects with the limbic system.

Fernstrom: Have you checked to see if the material you are measuring behaves like insulin in other systems?

Havrankova: Yes. So far, the material we find in brain behaves like insulin in the radioreceptor assay, the radioimmunoassay and the bioassay. Also, it separates the same as insulin on gel filtration.

Pardridge: Could you comment on the recent failure of Yalow's group to replicate your findings?

Havrankova: I think the differences may be methodological. For one thing, they extracted with acid-ethanol and then neutralized the extract directly in the assay. They didn't concentrate the material at all. They used different buffers. They also used a different species, but so far we have comparable results in rat and mouse, and Rosenzweig has found it in human brain as well. So I expect the differences are mainly methodological.

Bray: Have you tried immunofluorescence or any other technique for intracellular localization of insulin?

Havrankova: Yes, we have some preliminary data using immunocytochemistry. We find the insulin close to the nuclei in the olfactory bulb and cortex. We have not yet tried electron microscopy, but we can definitely say it is within the cell and near the Golgi area.

Bray: In starvation, brain metabolism changes to include the use of ketones. Is there a concomitant change of brain insulin?

Havrankova: This has not been determined.

Woods: Does streptozotocin cross the blood-brain barrier?

Havrankova: It's not supposed to cross. However, in order to control for that possibility, we gave streptozotocin directly to the CSF and found no change of brain insulin.

Oomura: For years, we have been studying cells in the lateral hypothalamus with the use of 7-barrelled micropipettes with attached intracellular recording electrodes. We find neurons which are hyperpolarized and have decreased spontaneous activity to the application of glucose, so we call them glucose-sensitive neurons. These glucose-sensitive neurons have insulin receptors since they increase their firing rate to the application of insulin in a dose-dependent manner. From the dose-response curve, we have calculated that one molecule of insulin reacts with one receptor site. More recently, we have studied insulin binding sites in rat whole brain preparations. We find that whereas the number of binding sites occupied by radiolabelled insulin and displaceable by cold insulin goes up in rat liver after treatment with streptozotocin or starvation for three days, there is no such change following either treatment on brain insulin receptors.
We also have found the highest concentrations of insulin receptors in the olfactory bulb and the hypothalamus. We find comparable levels in the olfactory bulb and the ventromedial and lateral hypothalamic areas. Further, the binding does not change in the hypothalamus following either starvation or treatment with streptozotocin. We also feel that insulin is synthesized within the brain.

Finally, we have recently found that glucagon decreases the activity of the glucose-sensitive neuron in the lateral hypothalamus.

Havrankova: I am pleased to see that your findings parallel ours so closely, especially your finding that brain insulin receptors do not seem to up- or down-regulate with a variety of treatments.

B. Jeanrenaud: What you really mean is that CNS insulin receptors do not change when changes occur in plasma insulin. You have no evidence that CNS insulin receptors do not down-regulate; one would have to vary brain insulin to determine that. In fact, in your hands, since insulin within the brain doesn't change with your various treatments, your finding that insulin receptors do not change is theoretically consistent with receptor regulation. Perhaps brain insulin receptors are continuously down-regulated by the very high levels of brain insulin you find.

Havrankova: You are right. All that we can say is that brain insulin receptors do not change with plasma insulin levels.

Porte: In your assay, do you find insulin in other tissues?

Havrankova: Yes, Rosenzweig finds very high levels in most tissues.

Porte: Could the insulin be localized to nerves within these tissues?

Havrankova: Possibly, but that would not explain the presence of insulin in circulating cells such as lymphocytes and monocytes.