Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 497 (2004) 105–110

www.elsevier.com/locate/ejphar

# Increased insulin is not required for $\beta_2$-adrenoceptor-induced increases in mouse brain tryptophan

Natalie R. Lenard*, Adrian J. Dunn

*Department of Pharmacology and Therapeutics and School of Graduate Studies, Louisiana State University Health Sciences Center, 1501 Kings Highway, Shreveport, LA 71130-3932, United States*

Received 11 June 2004; accepted 18 June 2004
Available online 20 July 2004

**Abstract**

The current study tested the hypothesis that $\beta_2$-adrenoceptor-mediated increases in brain tryptophan are caused by increased insulin secretion. Male mice were treated with streptozotocin (40 mg/kg) for 5 days to induce experimental diabetes. Control and diabetic mice were treated with the $\beta_2$-adrenoceptor agonist, clenbuterol (0.1 mg/kg), 1 h before selected brain regions were dissected for analysis by high performance liquid chromatography (HPLC) with electrochemical detection for tryptophan content, and plasma was collected for analysis of total and free tryptophan and glucose concentrations. Clenbuterol increased brain tryptophan and plasma glucose and decreased plasma total tryptophan but did not alter plasma free tryptophan. There were no significant differences in brain or plasma tryptophan between control and streptozotocin-treated mice. In a separate experiment, pretreatment of the mice with an insulin antibody did not prevent the clenbuterol-induced increases in brain tryptophan. These results suggest that $\beta_2$-adrenoceptor agonists increase brain tryptophan by a mechanism that does not involve changes in insulin.


*Keywords:* Insulin; Tryptophan; (Mouse); HPLC; β-Adrenoceptor

## 1. Introduction

Both exogenous insulin administration and endogenous insulin secretion are associated with significant elevations in brain tryptophan concentrations (De Montis et al., 1978; Fernstrom and Wurtman, 1971; MacKenzie and Trulson, 1978). Like insulin, β-adrenoceptor activation increases brain tryptophan concentrations. Under normal physiological conditions, brain concentrations of tryptophan are rate-limiting for serotonin (5-hydroxytryptamine, 5-HT) synthesis because tryptophan hydroxylase is not saturated by tryptophan (Eccleston et al., 1965; Fitzpatrick, 1999). Thus, increases in brain free tryptophan concentrations are likely to increase 5-HT synthesis (Moir and Eccleston, 1968; Poncet et al., 1993).

The nonselective adrenoceptor agonist, isoproterenol (Eriksson and Carlsson, 1988), the $\beta_2$-adrenoceptor selective agonist, clenbuterol (Edwards et al., 1989), and the $\beta_3$-adrenoceptor selective agonist CL 316243 (disodium 5-[(2*R*)-2-([(2*R*)-2-(3-chlorophenyl)-2-hydroxyethyl] amino) propyl]-1,3-benzodioxole-2,2-dicarboxylate) (Lenard et al., 2003) have been shown to induce robust increases in brain tryptophan in rodents. The $\beta_1$-adrenoceptor selective agonist, dobutamine, also increased brain tryptophan, but this effect was antagonized by a $\beta_2$-adrenoceptor selective antagonist (Lenard et al., 2003). Thus, it appears that $\beta_2$- and $\beta_3$-adrenoceptors, but not $\beta_1$-adrenoceptors, mediate increases in brain tryptophan. The mechanism may involve insulin secretion, because stimulation of β-adrenoceptors in pancreatic islet cells induces insulin secretion (Porte and Robertson, 1973). Because 5-HT dysregulation might play a role in the pathogenesis of depression, this disease might be more prevalent in diabetics; in fact, there is an increased risk of both type I and type II diabetics for unipolar depressed

* Corresponding author. Tel.: +1 318 675 4314; fax: +1 318 675 7857.
*E-mail address:* nlenar@lsuhsc.edu (N.R. Lenard).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.06.031

patients (McCarty, 1994). One study estimated the odds of depression in diabetes to be doubled, secondary to either the hardships of advancing diabetes or to diabetes-related changes in neurotransmitter function (Anderson et al., 2001).

The administration of streptozotocin to induce diabetes in laboratory animals has been used to examine insulin's role in physiologic processes (Dulin and Soret, 1977). The mechanism by which β-adrenoceptor agonists induce increases in mouse brain tryptophan is currently unknown. The current work was designed to test the hypothesis that $\beta_2$-adrenoceptor selective agonist-induced increases in mouse brain tryptophan are mediated by insulin. Thus the relationships between plasma glucose and brain tryptophan in both control and diabetic mice treated with β-adrenoceptor agonists were investigated.

## 2. Materials and methods

### 2.1. Animals

Three to five weeks old male CD-1 virus-antigen free (VAF plus) mice weighed 18–20 g on arrival (Charles River, Raleigh–Durham facility, Colony R16). Mice were initially group-housed in an AAALAC-accredited animal care facility at 22–23 °C under a 12:12-h light–dark cycle with the lights on at 0700 h. Food and water were available ad libitum. At least 48 h before each experiment, mice were placed in individual cages to avoid problems associated with disturbing group-housed animals. All procedures comply with the European Community guidelines for the use of experimental animals and were approved by the LSUHSC-S Animal Care and Use Committee.

### 2.2. Induction of experimental diabetes

Mice were injected intraperitoneally (i.p.) with streptozotocin (Sigma, St. Louis, MO) dissolved in citrate buffer (50 mM, pH 4.5) at a volume of 10 μl/g body weight every day for 5 consecutive days to induce experimental diabetes (Zielasek and Kolb, 1992). Control animals were injected with the citrate buffer alone. Hyperglycemia was verified at day 10 with the glucose assay (see below), and was considered to reflect a reduction in plasma insulin levels. However, plasma insulin measurements were not performed, because the available plasma was used for glucose and tryptophan assays.

### 2.3. Experimental procedures

Mice were injected i.p. with saline or clenbuterol (4-amino-α-(t-butylaminomethyl)-3,5-dichlorobenzyl alcohol hydrochloride, Sigma/RBI, Natick, MA) dissolved in 0.9% sterile saline at a volume of 10 μl/g body weight. Mice were sacrificed by decapitation one h following the last injection, and trunk blood was collected into Eppendorf tubes containing 20 μl 1.5 M EDTA-$Na_2$. The brain was rapidly removed and frontal cortex, hypothalamus, and brain stem were dissected (Dunn, 1988). Brain regions were weighed in tared Eppendorf tubes and frozen on dry ice. The frozen samples were homogenized by ultrasonication in 0.1 M $HClO_4$ containing 0.1 mM EDTA and an internal standard of *N*-methyldopamine. The homogenates were centrifuged for four min and the supernatants were analyzed by HPLC with electrochemical detection, similar to what was described previously (Dunn, 1993). Briefly, a shortenend HPLC run was used to measure tryptophan only (retention time 7.5 min). For this, we used a Spherisorb octadecyl silane (ODS 1) reverse-phase column (25 cm, 5 μm, Keystone Scientific, Bellefonte, PA) shortened to 12.5 cm. The mobile phase contained 0.05 M $NaH_2PO_4$ (pH 2.75), 0.1 mM EDTA, 0.5 mM octanesulfonic acid (sodium salt), and 5% acetonitrile. Plasma was separated by centrifugation for 15 min at 4 °C and frozen at −70 °C until assayed for glucose or tryptophan. In the antibody experiments, the guinea pig anti-porcine insulin antibody and the nonimmune guinea pig serum (both from Sigma) were diluted with 0.9% sterile saline such that 300 μl of the dilute solution contained 50 μl of the original solution. It has been shown that C57BL/6J mice treated with guinea pig anti-porcine insulin antibody show impaired glucose tolerance following an i.p. glucose challenge, indicating a lack of normal insulin activity (Loftus et al., 1998). Mice were injected i.p. with 300 μl of either solution one h before saline or clenbuterol.

### 2.4. Plasma tryptophan analysis

To measure total tryptophan, 50 μl of plasma were deproteinized with 50 μl of 0.2 M $HClO_4$ containing 100 ng/ml *N*-methyldopamine. To measure free tryptophan, 125 μl of fresh plasma was transferred to an ultrafiltration tube (Ultrafree-MC, Millipore, Bedford, MA) with a 30,000 nominal molecular weight limit low binding regenerated cellulose filter and centrifuged for 15–20 min at 4500×*g* at room temperature. Thirty-five microliters of the ultrafiltrate was deproteinized with 35 μl of 0.2 M $HClO_4$ containing 100 ng/ml *N*-methyldopamine. Very little, if any, visible protein was precipitated, indicating that albumin did not pass through the filter. Ten microliters of the total tryptophan dilution and 35 μl of the ultrafiltrate dilution were injected into the HPLC as described above.

### 2.5. Plasma glucose determination

Plasma glucose was determined colorimetrically at 490 nm with a commercially available glucose (trinder) test kit (Sigma) using glucose (0–750 mg/dl) as the standard.

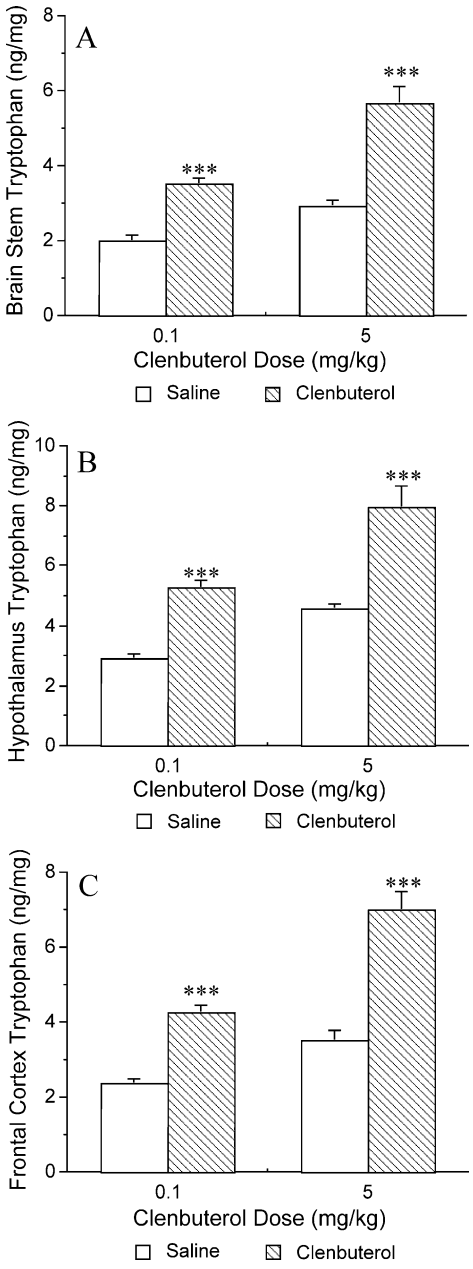


Fig. 1. Effect of clenbuterol on (A) brain stem, (B) hypothalamus and (C) frontal cortex tryptophan concentrations. Mice ($n$=6) were injected with saline or clenbuterol (0.1 and 5 mg/kg, i.p.) and brain and blood samples collected 60 min later. ***Significantly different from saline-treated animals in the same group, $P$<0.001.

### 2.6. Statistical analysis

Data are represented as means±standard error of the mean (S.E.M). Two-way analysis of variance (ANOVA) was performed to determine main effects and interactions between glycemic status and drug treatments. Fisher's least significant difference (LSD) test was used for individual comparisons. For comparisons between two groups, Students two-tailed $t$-test was used. Statistical significance was accepted at $P$<0.05.

## 3. Results

### 3.1. Effect of clenbuterol on brain tryptophan

Mice were treated with saline or one of two doses of clenbuterol. Both doses significantly elevated hypothalamus tryptophan ($P$<0.001). These data were derived from two separate experiments performed on different days, which may explain why the saline-injected control values are significantly different (Fig. 1).

### 3.2. Effect of clenbuterol and streptozotocin on plasma glucose

Mice were treated with streptozotocin (40 mg/kg) for 5 days to induce experimental diabetes. Control and streptozotocin mice were treated with saline or clenbuterol (0.1 mg/kg) 60 min before brain and blood samples were collected. Streptozotocin-treated animals also had significantly elevated plasma glucose concentrations, verifying the induction of diabetes ($F$(1,24)=59.1, $P$<0.001). Clenbuterol significantly elevated plasma glucose in both control and streptozotocin-treated animals (Fig. 2, $F$(1,24)=9.70, $P$<0.01).

### 3.3. Effect of clenbuterol on brain tryptophan content

To determine whether insulin plays a role in clenbuterol-induced increases in brain tryptophan, hypothalamus tryptophan content was determined in the same animals. Clenbuterol significantly elevated hypothalamus tryptophan content in both control and streptozotocin-treated animals (Fig. 3). Two-way ANOVA revealed no interaction between glycemic status and clenbuterol treatment ($F$(1,24)=0.018, $P$=0.89) but did reveal a main effect of

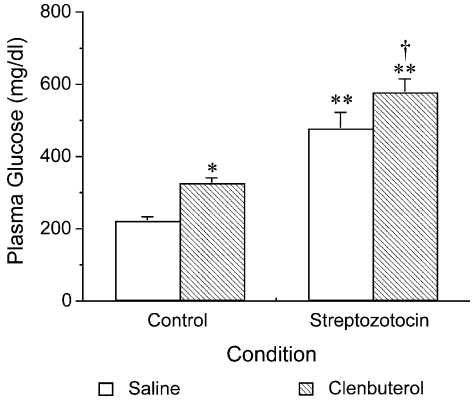


Fig. 2. Plasma glucose concentrations in saline- and clenbuterol-treated control and diabetic mice. Mice ($n$=5–7) were injected with 40 mg/kg streptozotocin in citrate buffer or the citrate buffer alone (control) for 5 consecutive days. On day 15, mice were injected with saline or clenbuterol (0.1 mg/kg) and brain and blood samples collected 60 min later. *Significantly different from saline-control, $P$<0.05, **$P$<0.01. †Significantly different from streptozotocin-saline, $P$<0.05.

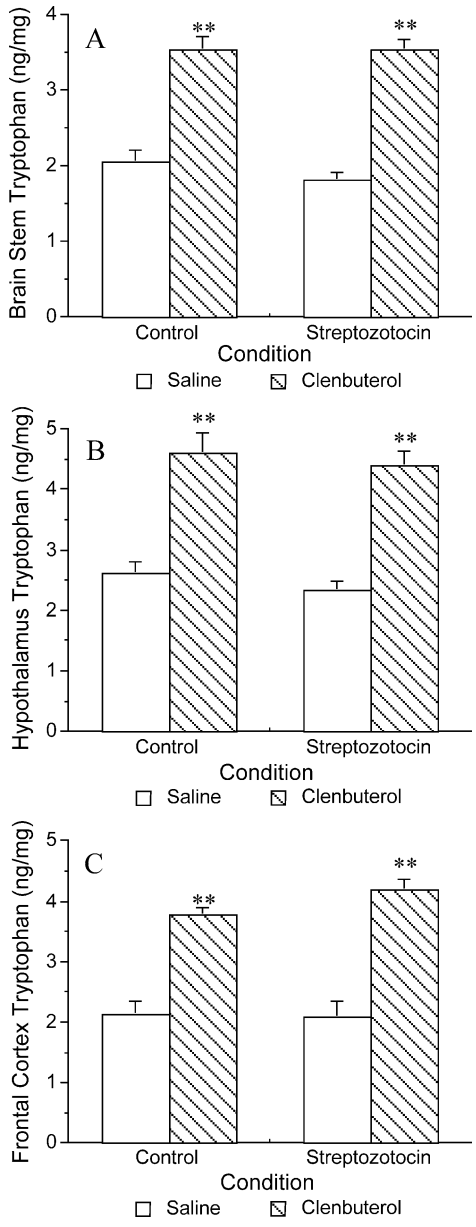


Fig. 3. (A) Brain stem, (B) hypothalamus and (C) frontal cortextryptophan concentrations in saline- and clenbuterol-treated control and streptozotocin-treated mice. Brain samples were collected from the same animals used for Fig. 2. **Significantly different from saline-treated animals in the same group, $P<0.01$.

clenbuterol treatment ($F(1,24)=72.6$, $P<0.001$). Similar results were obtained in brain stem (interaction: $F(1,24)=0.834$, $P=0.37$; main effect: $F(1,24)=88.1$, $P<0.001$) and frontal cortex (interaction: $F(1,24)=0.583$, $P=0.45$; main effect: $F(1,24)=116$, $P<0.001$).

### 3.4. Effect of clenbuterol on plasma tryptophan concentrations

Previous studies in rats have shown that insulin administration elevates plasma total tryptophan and decreases plasma free tryptophan (Fernando et al., 1976; Fernstrom and Wurtman, 1972). If clenbuterol-induced increases in brain tryptophan are mediated by insulin, it is likely that similar effects would be observed on plasma tryptophan. Plasma tryptophan was significantly decreased by clenbuterol in both control and streptozotocin-treated animals (Fig. 4A). No significant interaction between glycemic status and clenbuterol treatment was detected by two-way ANOVA ($F(1,23)=8.68$, $P=0.55$). Plasma free tryptophan was not altered (Fig. 4B).

### 3.5. Effects of insulin antibodies on clenbuterol-induced increases in brain tryptophan

It has been shown that C57BL/6J mice treated with guinea pig anti-porcine insulin antibody show impaired glucose tolerance following an i.p. glucose challenge, indicating a lack of normal insulin activity (Loftus et al., 1998). To further assess the role of insulin in the clenbuterol-induced increases in brain tryptophan, mice were injected with guinea pig anti-porcine insulin antibody or guinea pig serum 60 min before saline or clenbuterol and brain and blood samples were collected 60 min later.

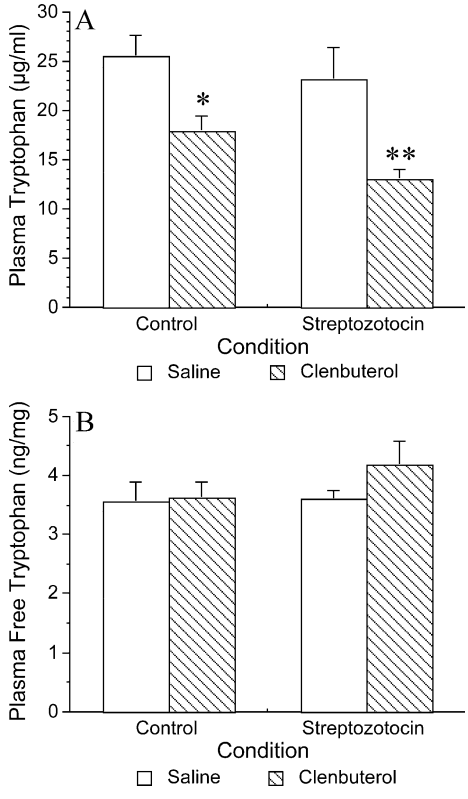


Fig. 4. Plasma (A) total and (B) free tryptophan concentrations in saline- and clenbuterol-treated control and diabetic mice. Plasma from the mice used in Figs. 1 and 2 was analyzed. Total and free tryptophan values represent pre- and post-ultrafiltration concentrations, respectively. *Significantly different from saline-treated animals in the same group, $P<0.05$, **$P<0.01$.

Clenbuterol treatment significantly elevated plasma glucose. Plasma glucose in control mice (i.e. without clenbuterol) pretreated with the insulin antibody was not significantly different from that in control mice pretreated with guinea pig serum (data not shown). Clenbuterol significantly increased hypothalamus tryptophan in both guinea pig serum- and insulin antibody-pretreated animals (Fig. 5). Two-way ANOVA revealed no interaction between pretreatment and agonist treatment ($F(1,21)=1.09$, $P=0.31$). Similar results were obtained in brain stem ($F(1,21)=4.34$, $P=0.054$) and frontal cortex ($F(1,21)=3.95$, $P=0.062$)).

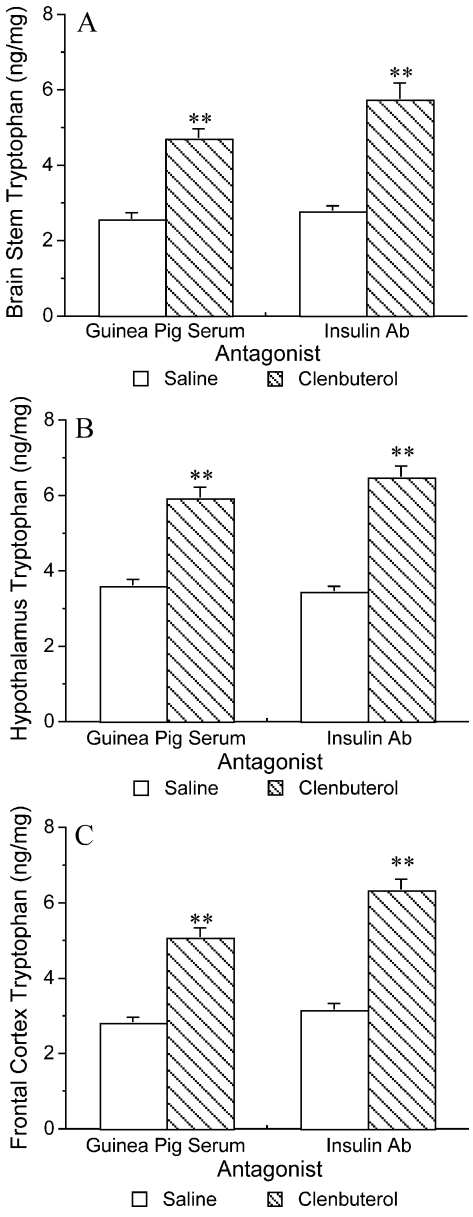


Fig. 5. Effect of insulin antibody on clenbuterol-induced increases in (A) brain stem, (B) hypothalamus and (C) frontal cortex tryptophan concentrations. Mice ($n=6$) were injected with 50 μl guinea pig serum or 50 μl insulin antibodies 60 min before saline or clenbuterol (0.1 mg/kg) and brain and blood samples collected 60 min later. **Significantly different from saline-treated animals in the same group, $P<0.01$.

## 4. Discussion

The present results suggest that activation of $\beta_2$-adrenoceptors by clenbuterol increased mouse brain tryptophan independently of insulin secretion. Clenbuterol induced robust increases in brain tryptophan in both control and streptozotocin-treated mice (Fig. 3). Streptozotocin is a toxin that destroys pancreatic beta cells selectively (Dulin and Soret, 1977); thus, presumably the streptozotocin-treated mice produced no insulin. The lack of effect of insulin antibody pretreatment on clenbuterol-mediated increases in brain tryptophan (Fig. 5) provided further evidence to dissociate the effects of clenbuterol on insulin release and brain tryptophan.

Clenbuterol treatment also decreased plasma total tryptophan but did not alter plasma free tryptophan. Previous studies have demonstrated that insulin administered to fasted rats *increased* plasma total tryptophan (Fernstrom and Wurtman, 1972) and decreased plasma free tryptophan (Fernando et al., 1976). Feeding carbohydrates to rats (which stimulates the release of endogenous insulin) had comparable effects on plasma total tryptophan (Fernstrom and Wurtman, 1971). Furthermore, insulin administration elevates brain tryptophan in fasted (Fernstrom and Wurtman, 1971), but not in fed rats (Curzon and Knott, 1974), whereas the mice used in the current study were fed ad libitum. Thus, clenbuterol produced effects on plasma tryptophan that are unlike those observed in insulin-treated animals.

It is possible that treatment of mice with streptozotocin or the insulin antibody at the doses used here did not sufficiently impair insulin function. If clenbuterol-induced increases in brain tryptophan requires only small amounts of insulin to be secreted, it is plausible, but unlikely, that insulin mediated the effect of clenbuterol on brain tryptophan.

Taken together, the above results suggest that insulin is not a significant mediator of β-adrenoceptor-stimulated increases in mouse brain tryptophan. Because clenbuterol decreased total tryptophan but did not affect free tryptophan, the mechanism by which clenbuterol increases brain tryptophan might involve the concurrent alteration of plasma total tryptophan. Further experimentation will be required to elucidate the relationships between plasma total and free tryptophan and brain tryptophan in β-adrenoceptor agonist-treated animals.

## Acknowledgements

We thank Glenn Farrar and Charles Dempsey for their technical assistance. This work was supported by National Institutes of Health Grant MH50947.

## References


Anderson, R.J., Freedland, K.E., Clouse, R.E., Lustman, P.J., 2001. The prevalence of comorbid depression in adults with diabetes: a meta-analysis. Diabetes Care 24, 1069–1078.

Curzon, G., Knott, P.J., 1974. Effects on plasma and brain tryptophan in the rat of drugs and hormones that influence the concentration of unesterified fatty acid in the plasma. Br. J. Pharmacol. 50, 197–204.

De Montis, M.G., Olianas, M.C., Haber, B., Tagliamonte, A., 1978. Increase in large neutral amino acid transport into brain by insulin. J. Neurochem. 30, 121–124.

Dulin, W.G., Soret, M.G., 1977. Chemically and hormonally induced diabetes. In: Volk, B.W., Wellman, K.F. (Eds.), The Diabetic Pancreas. Plenum, New York, pp. 425–465.

Dunn, A.J., 1988. Stress-related changes in cerebral catecholamine and indoleamine metabolism: lack of effect of adrenalectomy and corticosterone. J. Neurochem. 51, 406–412.

Dunn, A.J., 1993. Neurochemical methods for evaluating cerebral biogenic amine responses to cytokines and their involvement in the central actions of interleukin-1. In: De Souza, E.B. (Ed.), Neurobiology of Cytokines, vol. 17. Part B. Academic Press, San Diego, pp. 209–222.

Eccleston, D., Ashcroft, G.W., Crawford, T.B., 1965. 5-hydroxyindole metabolism in rat brain. A study of intermediate metabolism using the technique of tryptophan loading. II. Applications and drug studies. J. Neurochem. 12, 493–503.

Edwards, D.J., Sorisio, D.A., Knopf, S., 1989. Effects of the beta 2-adrenoceptor agonist clenbuterol on tyrosine and tryptophan in plasma and brain of the rat. Biochem. Pharmacol. 38, 2957–2965.

Eriksson, T., Carlsson, A., 1988. Beta-adrenergic control of brain uptake of large neutral amino acids. Life Sci. 42, 1583–1589.

Fernando, J.C.R., Knott, P.J., Curzon, G., 1976. The relevance of both plasma free tryptophan and insulin to rat brain concentration. J. Neurochem. 27, 343–345.

Fernstrom, J.D., Wurtman, R.J., 1971. Brain serotonin content: increase following ingestion of carbohydrate diet. Science 174, 1023–1025.

Fernstrom, J.D., Wurtman, R.J., 1972. Elevation of plasma tryptophan by insulin in the rat. Metabolism 21, 337–342.

Fitzpatrick, P.F., 1999. Tetrahydropterin-dependent amino acid hydroxylases. Annu. Rev. Biochem. 68, 355–381.

Lenard, N.R., Gettys, T.W., Dunn, A.J., 2003. Activation of beta(2)- and beta(3)-adrenergic receptors increases brain tryptophan. J. Pharmacol. Exp. Ther. 305, 653–659.

Loftus, T.M., Kuhajda, F.P., Lane, M.D., 1998. Insulin depletion leads to adipose-specific cell death in obese but not lean mice. Proc. Nat. Acad. Sci. U. S. A. 95, 14168–14172.

McCarty, M.F., 1994. Enhancing central and peripheral insulin activity as a strategy for the treatment of endogenous depression—an adjuvant role for chromium picolinate? Med. Hypotheses 43, 247–252.

MacKenzie, R.G., Trulson, M.E., 1978. Effects of insulin and streptozotocin-induced diabetes on brain tryptophan and serotonin metabolism in rats. J. Neurochem. 30, 205–211.

Moir, A.T., Eccleston, D., 1968. The effects of precursor loading in the cerebral metabolism of 5-hydroxyindoles. J. Neurochem. 15, 1093–1108.

Poncet, L., Denoroy, L., Jouvet, M., 1993. Daily variations in in vivo tryptophan hydroxylation and in the contents of serotonin and 5-hydroxyindoleacetic acid in discrete brain areas of the rat. J. Neural. Transm. Gen. Sect. 92, 137–150.

Porte Jr., D., Robertson, R.P., 1973. Control of insulin secretion by catecholamines, stress, and the sympathetic nervous system. Fed. Proc. 32, 1792–1796.

Zielasek, J., Kolb, H., 1992. Ganglioside therapy of type I diabetes: enhancement of hyperglycemia in the low dose streptozotocin model. Life Sci. 51, 49–52.