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Effects of streptozotocin-induced diabetes mellitus and insulin treatment on neuropeptide Y mRNA in the rat hypothalamus*

J. L. Marks, K. Waite, M. Li

Department of Clinical Endocrinology, Westmead Hospital, Westmead, Australia

Summary. Levels of neuropeptide Y and neuropeptide Y mRNA are increased in the arcuate nucleus of severely diabetic rats which may be the result of the associated marked hypoinsulinaemia. We hypothesised that if neuropeptide Y mRNA is regulated by physiological changes in circulating insulin, then the relatively minor changes in circulating insulin found in mild diabetes would also affect neuropeptide Y expression and its response to changing insulin levels should be rapid. Neuropeptide Y mRNA was quantified by in situ hybridisation through the rostral, mid and caudal levels of the arcuate nucleus of adult female rats. Neuropeptide Y mRNA was significantly increased at all three levels of the arcuate nucleus, 7 days after i.v. administration of 40 mg/kg streptozotocin. Neuropeptide Y mRNA was not further increased in the arcuate nucleus of animals given 50 mg/kg streptozotocin. In the former group, serum glucose was increased but insulin levels and body weights were the same as in control rats. In the 50 mg/kg streptozotocin group, serum glucose was further increased while serum insulin and body weight were reduced. In addition, neuropeptide Y mRNA was not altered in the hypothalamic dorsomedial nucleus or the thalamic reticular nucleus. When diabetic rats were treated for 20 h with s.c. insulin, there was decreased neuropeptide Y mRNA in the arcuate nucleus. We conclude that neuropeptide Y mRNA in the arcuate nucleus is responsive to small changes in circulating insulin levels and the response occurs within 20 h. These data support that circulating insulin may contribute to control of neuropeptide Y expression under physiological conditions.

Key words: Neuropeptide Y, diabetes mellitus, insulin action, hypothalamus, neuropeptides.

Neuropeptide Y (NPY) is a 36 amino acid neuropeptide [1] that is found abundantly in the mammalian brain [2]. In the hypothalamus, NPY has potent effects on food and water consumption [3, 4] and reproductive functions [5]. Nerve terminals containing NPY are widely distributed in the hypothalamus, especially in the paraventricular nucleus (PVN) and originate in neuronal cell bodies in the arcuate nucleus [6] and the brainstem [7]. The distribution of NPY and its ability to stimulate food intake when administered into the third ventricle [3], PVN or adjacent nuclei [4], suggests that NPY has an important role in controlling food intake and body weight in vivo. In fasted rodents, NPY is increased in the PVN and to a lesser extent in other hypothalamic nuclei [8, 9] while NPY mRNA is increased in the arcuate nucleus [10–12]. Release of NPY in the PVN is also increased in fasted animals and decreases after food intake [13].

In diabetic rodents, NPY [14–16] and NPY mRNA [17, 18] are also increased in several hypothalamic sites and arcuate nucleus, respectively. As both diabetes and fasting are hypoinsulinaemic states, it has been proposed that circulating insulin decreases the expression of NPY in the hypothalamus. However, in addition to low circulating insulin, these metabolic states have many other similarities that could potentially affect NPY expression. Recently it has been shown that administration of insulin into the third ventricle reduces NPY mRNA in the arcuate nucleus and NPY immunoactivity in the PVN of fasted rats [19, 20]. This suggests that there is a direct effect of insulin on hypothalamic NPY expression.

Although studies using diabetic rats have provided some of the strongest support for the hypothesis that circulating insulin modulates hypothalamic NPY expression, the majority of these studies have been performed following prolonged periods of severe diabetes. From these data it is not clear if the changes in NPY expression were due to the reduced insulin levels, weight loss, metabolic consequences of diabetes [21] or combinations of these factors.

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Furthermore the time course of the diabetic effect on NPY expression has not been defined. Consequently, in diabetic animals, it is unknown if the hypoinsulinaemia directly, subsequent metabolic events, or both, affect hypothalamic NPY expression.

To determine if low insulin levels directly increase hypothalamic NPY expression, mildly diabetic animals with minimal metabolic consequences of their hypoinsulinaemia could be used. If reduced insulin levels produced by mild diabetes are similar to the physiological low insulin levels that occur prior to the active period [22] this would suggest that insulin modulates hypothalamic NPY under normal conditions. This would require that the insulin effect on NPY expression was direct and occurred relatively rapidly.

The aim of this study was to determine the response of hypothalamic NPY expression to both minor and more severe insulin deficiency using a diabetic model. Different doses of streptozotocin were administered to rats to induce mild and more severe diabetes, while NPY expression was assessed by measuring NPY mRNA in consecutive levels through the hypothalamus. We have also determined how rapidly insulin treatment affects NPY mRNA in the same model.

Materials and methods

Adult female Wistar rats, aged 12–14 weeks and weighing 195–256 g were used in these studies. They were housed 3–4 in plastic cages, fed ad libitum on Purina rat chow and maintained on a 12-h light and dark cycle beginning at 06.00 hours. Diabetes was induced with i.v. streptozotocin (Sigma, St. Louis, Mo., USA) in 0.9% NaCl containing 20 mmol/l Na citrate, pH 5.0. Controls were injected with vehicle alone. Animals were returned to individual cages and urine monitored for development of glycosuria using Keto-Diastix (Bayer Diagnostics, Mulgrave, Vic., Australia). Animals were excluded from the study if more than a trace of ketones was detected or if they did not develop glycosuria. Generally glycosuria developed 3–4 days after streptozotocin treatment. In experiment 1, 7 days after the i.v. injections, animals were killed or in experiment 2, they were treated with s.c. insulin.

In experiment 2, 7 days after treatment with 50 mg/kg streptozotocin, animals with confirmed diabetes were divided into three groups. Group 1 was killed at 13.00 hours on the same day while groups 2 and 3 were started on 3 IU human ultralente insulin (CSL-Novo, North Rocks, NSW, Australia) at 17.00 hours and human lente insulin (CSL-Novo) 1.5 IU at 08.00 hours. Group 2 was killed 24 h after Group 1 i.e. 20 h after the first insulin injection and Group 3, 48 h after Group 2.

In all experiments, animals were killed under i.p. rompun/ketamine anaesthesia. Blood was collected from the inferior vena cava, followed by perfusion of the brain via the left cardiac ventricle with 30 ml of ice-cold 0.9% NaCl. Brains were rapidly removed, frozen on dry ice and stored at −70 °C.

Coronal sections (15 μm) were cut through the arcuate nucleus on a cryostat. We have previously reported on NPY mRNA expression in five different levels through the rostro-caudal axis of the arcuate nucleus [12]. In this study, we have only used slices from levels through the arcuate nucleus that contained relatively large amounts of NPY mRNA. These caudal, mid and rostral levels were equivalent to Bregma −3.6 mm, Bregma −3.3 and Bregma −2.8 mm, respectively and were based on their similarity to figures in a stereotaxic atlas [23]. Twelve adjacent slices were cut at each of these levels and used for in situ hybridization or representative slices from each region of each animal were stained with cresyl violet to confirm the correct localization of slices to the expected level.

Table 1. Metabolic effects of streptozotocin treatment, Experiment 1 (see Materials and methods for details)

Groups	Change body weight (%)	Serum glucose (mmol/l)	Serum insulin (μU/ml)	Food intake (g/day)
Control (8)	3.2 ± 0.4[a]	12.6 ± 0.6	7.8 ± 0.8	22 ± 7
Streptozotocin, 40 mg/kg (5)	2.8 ± 1.5[a]	21.8 ± 5.7[b]	7.2 ± 2.8	32 ± 6
Streptozotocin, 50 mg/kg (4)	−3.9 ± 1.4[a]	34.8 ± 0.8[b]	3.3 ± 0.8[b]	35 ± 0[b]

Values presented are the mean ± SEM. The number of animals in each group is in parentheses. The change in body weight is the percentage change from before streptozotocin or vehicle treatment to the time of killing. [a] $p < 0.05$ compared to initial weight, [b] $p < 0.05$ compared to control values

Quantitative in situ hybridization

In situ hybridization for NPY mRNA was performed as previously described [12, 19]. The probe used was a 36 base oligonucleotide, previously described [19] and 3′ end-labelled with ^{35}S dATP. Following hybridization, the sections were autoradiographed on Hyperfilm β-max film (Amersham, North Ryde, NSW, Australia) for 4 days. Film images were analysed using the JAVA image analysis system (Jandel Scientific, Corte Madera, Calif., USA), standardized with ^{14}C plastic sections (Amersham) and the total NPY mRNA signal in bilateral arcuate nuclei was quantified as previously described [12]. Four brain slices from each arcuate level from each animal were analysed. NPY mRNA is measured in arbitrary units but can be compared between the experiments.

Total NPY mRNA signal in the hypothalamic dorsomedial nucleus was analysed in the same way and on the same rostral or mid level autoradiographs used for measuring NPY mRNA in the arcuate nucleus. Because the expression of NPY mRNA in the dorsomedial nucleus occurs over a narrow length of the rostro-caudal axis, 2–4 slices from each animal were used to measure NPY mRNA in this nucleus. NPY mRNA signal in the reticular nucleus of the thalamus was analysed as a density only because of the large and irregular size of this nucleus. Two density measurements were made from a mid and from a rostral level slice from each animal and background density as described above was subtracted.

Serum measurements

Serum insulin and serum glucose were measured as previously described [24]. The insulin RIA was performed with human standards and it has been previously found that human and rat serum have similar dilution curves with this assay [24]. The sensitivity of the insulin assay was 2 μU/ml.

Statistical analysis

All data are presented as the mean ± SEM. The values for NPY mRNA in each level through the arcuate nucleus have been calculated from the total number of brain slices in that group and not from the number of animals. Because considerable variation exists in the level of NPY mRNA in the arcuate nucleus of different animals, statistical comparisons were made with a non-parametric test, the Mann-Whitney U test. The results for NPY mRNA in all levels of the arcuate nucleus of one animal in Group 2, experiment 2 were excluded from analysis because they were all higher than the mean plus 3 SD for all the other animals in that group.

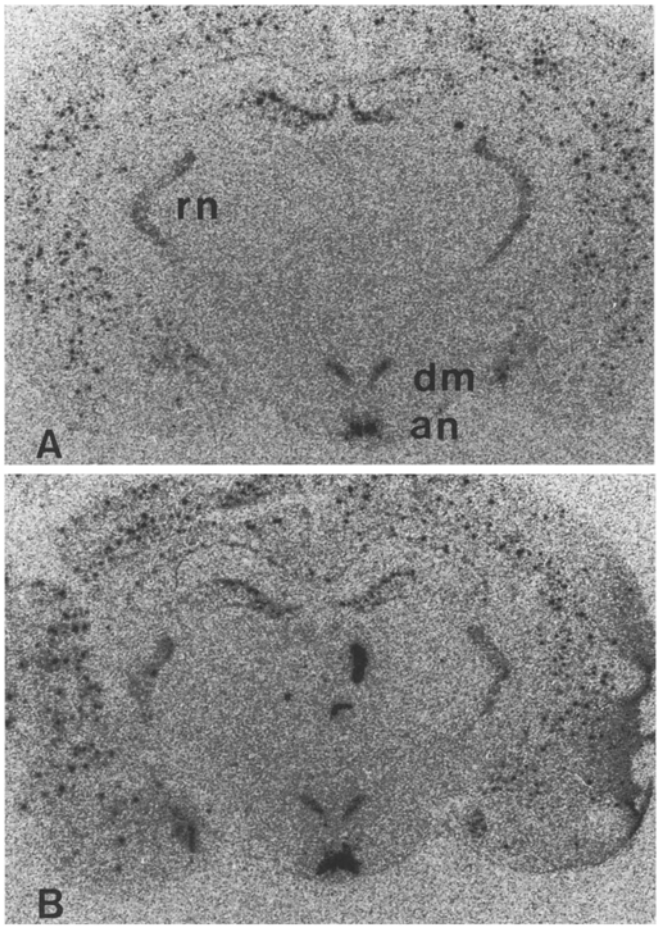

Fig. 1 A, B. In situ hybridization autoradiographs of neuropeptide Y mRNA in coronal brain sections through the mid level (see Materials and methods) of the arcuate nucleus. **A** is from a control animal and **B** from an animal treated with 50 mg/kg streptozotocin. Two dark spots in the centre of the brain slice in **B** are artifactual. an, Arcuate nucleus; dm, dorsomedial nucleus of the hypothalamus; rn, reticular nucleus of the thalamus

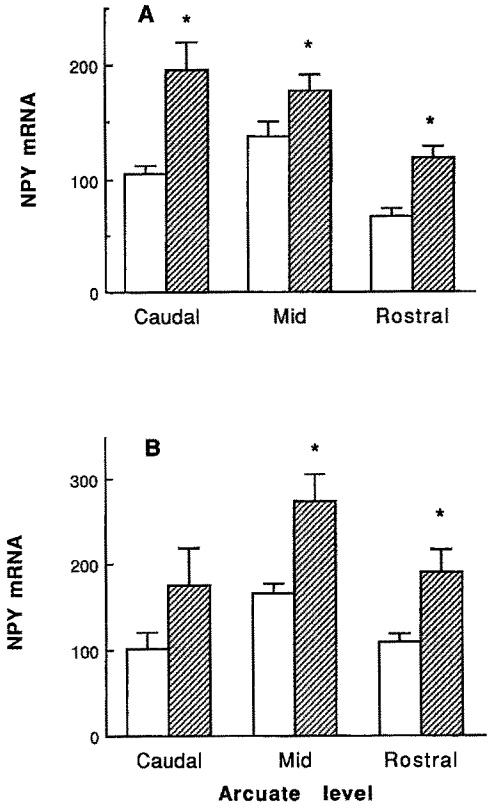

Fig. 2 A, B. The effect of streptozotocin on neuropeptide Y mRNA in the caudal, mid and rostral levels of the arcuate nucleus. Results are the mean ± SEM for total NPY mRNA signal in bilateral arcuate nuclei and were determined from in situ hybridization autoradiographs as described in Materials and methods. Values for NPY mRNA are in arbitrary units. **A,** Six adult female rats were injected with vehicle or 40 mg/kg streptozotocin. **B,** Six adult females were injected with vehicle or 50 mg/kg streptozotocin. Control rats □, diabetic rats ▨. * $p < 0.05$ compared to the control rats

Results

The effect of 40 and 50 mg/kg streptozotocin on body weight, food intake, serum glucose and insulin concentrations are shown in Table 1. Control animals gained weight during the study as did those treated with 40 mg/kg streptozotocin, while animals treated with 50 mg/kg had a decrease in body weight. Food intake was increased in both diabetic animal groups, significantly for the 50 mg/kg dose and was similar for the two doses of streptozotocin. Serum glucose values were increased in 40 mg/kg treated animals and were further increased with the higher dose while serum insulin was decreased only with the higher dose of streptozotocin.

The distribution of NPY mRNA in a coronal brain section at the mid level through the arcuate nucleus is shown in Figure 1. NPY mRNA was expressed in many brain regions and was particularly prominent in the arcuate nucleus. In a similarly located slice from a diabetic animal, denser signal was apparent in the arcuate nucleus but not in other regions. The effect of diabetes induced with 40 mg/kg of streptozotocin on NPY mRNA expression in the arcuate nucleus is quantified in Figure 2A. At the caudal, mid and rostral levels, NPY mRNA was increased by 87, 29 and 76%, respectively. The effect of 50 mg/kg of streptozotocin is shown in Figure 2B. NPY mRNA was increased in the mid and rostral levels by 64 and 75%, respectively. A similar increase was found in the caudal level but this failed to reach significance. Compared to the lower dose, there was possibly a further increase in NPY mRNA due to the higher dose of streptozotocin only in the mid level of the arcuate nucleus (64 ± 20% vs 29 ± 11% increase, $p = 0.06$). On the other hand, in both the dorsomedial nucleus of the hypothalamus and the reticular nucleus of the thalamus, 50 mg/kg streptozotocin had no effect on NPY mRNA (Fig. 3).

The effect of administering insulin to diabetic animals is shown in Figure 4. After 20 h of insulin treatment, NPY mRNA had decreased in the caudal and mid levels of the arcuate nucleus. There was little or no change after a further 48 h of treatment at these levels but in the rostral level with prolonged treatment, a decrease in NPY mRNA was seen. The metabolic effects of insulin treatment are shown in Table 2. There was an increase in serum insulin, a ten-

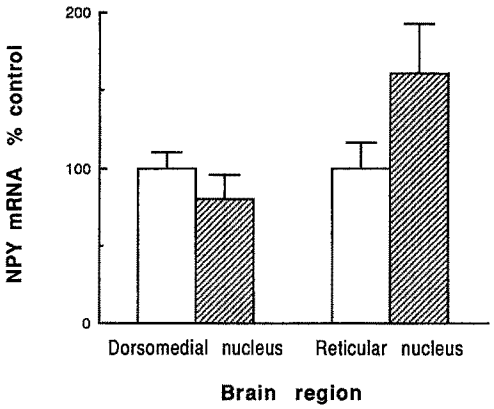

Fig. 3. The effect of 50 mg/kg streptozotocin on neuropeptide Y mRNA in the reticular nucleus of the thalamus and the dorsomedial nucleus of the hypothalamus. Measurements are the mean ± SEM for the same autoradiographs used in Figure 2. Measurements of reticular nucleus NPY mRNA were on one mid and one rostral level slice from four fasted and four diabetic rats. Measurements of dorsomedial nucleus NPY mRNA were on mid or rostral level autoradiographs. Two to four measurements were taken from each of six control □ and diabetic ▨ rats

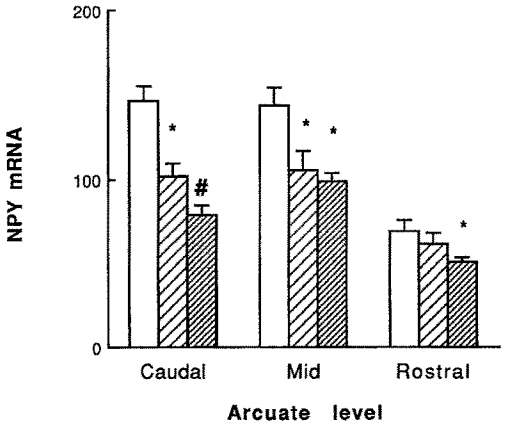

Fig. 4. The effect of s.c. insulin on neuropeptide Y mRNA in the arcuate nucleus of diabetic animals. There were six animals in the no insulin group □ and five animals each in the group treated with insulin for 20 h ▨ and for 68 h ▨. * $p < 0.05$ compared to the no insulin group, # $p < 0.05$ compared to the 20-h insulin treated group

dency for serum glucose to decrease but no effect on body weight. Similarly there was no effect on food intake i.e. 29 ± 4 g/day before and 28 ± 4 g/day after insulin for 20 h.

Discussion

Previous studies have shown increased hypothalamic NPY peptide [14–16] and mRNA [17, 18] in diabetic rodents. In these studies, the animals were diabetic for a considerable period, had significant weight loss and marked hyperglycaemia. For example, in the study of White et al. [17], 17–19 days after streptozotocin treatment, NPY mRNA was increased in the arcuate nucleus. At killing, animals had lost 44% of their body weight and had blood glucose levels three times higher than controls. In the study of Abe et al. [16], NPY levels were increased in the

Table 2. Metabolic effects of s.c. insulin injections in diabetic animals, Experiment 2 (see Materials and methods for details)

	Change body weight (%)	Serum glucose (mmol/l)	Serum insulin (μU/ml)
Group 1	−6.2 ± 8.3	23.2 ± 0.5	7.0 ± 0.8
Group 2	−1.2 ± 7.3	18.6 ± 2.2	15.8 ± 3.8[a]
Group 3	−2.2 ± 5.6	20.1 ± 1.7	22.1 ± 7.2[a]

Group 1 animals did not receive insulin, Group 2 received insulin for 20 h and Group 3 received insulin for 68 h. The change in body weight is the same as in Table 1. [a] $p < 0.05$ compared to values in Group 1. Each group contained 4 or 5 animals

PVN and arcuate nucleus of BBW rats, 23 weeks after development of glycosuria, but they weighed 20% less than controls and had blood glucose levels which were three times higher. Although these studies suggest a relationship between insulin levels and NPY expression, the associated metabolic, hormonal and weight changes are confounding factors.

In this study, we have demonstrated that NPY mRNA expression in the arcuate nucleus is increased by streptozotocin-diabetes, of similar severity to previous studies. The animals treated with 50 mg/kg streptozotocin had a 50% reduction in serum insulin, a three-fold increase in serum glucose as well as a 4% weight loss. NPY mRNA was increased in the mid and rostral levels of the arcuate nucleus 3–4 days after the onset of glycosuria. This effect occurred much sooner after development of diabetes than previously reported [14–18]. By contrast, in the dorsomedial nucleus, despite its proximity to the arcuate nucleus and also in the reticular nucleus of the thalamus, there was no effect of 50 mg/kg streptozotocin on NPY mRNA. It has to be considered that small changes in NPY mRNA produced by streptozotocin in these nuclei could have been missed and may have been more obvious with more severe or prolonged hypoinsulinaemia. Nevertheless, White et al. [17] found that diabetes had no effect on NPY mRNA in the reticular nucleus of the thalamus and cerebral cortex of rats, while we have previously reported that NPY mRNA in the dorsomedial nucleus and thalamus is unaffected by fasting [12] despite large increases in the arcuate nucleus. Thus, changes in NPY mRNA in both these metabolic states appear to be localised to the arcuate nucleus. The reason that NPY-ergic neurons in this region of the hypothalamus are sensitive to the changes produced by these states is unclear but may be due to the proximity of the median eminence and the access to peripheral signals.

The reported values for serum glucose in control animals were relatively high, probably due to stress of anaesthesia (unpublished data). In animals given 40 mg/kg streptozotocin, there was a less than two-fold increase in serum glucose but no effect on body weight or serum insulin. Despite these minor metabolic effects, there was increased NPY mRNA in all levels of the arcuate nucleus tested, approaching that found with the higher dose of streptozotocin. The presence of hyperglycaemia confirms that there was some degree of insulin deficiency, despite the lack of a significant decrease in insulin levels. This may have several explanations. Firstly, in control rats, insulin levels may have been close to trough values as blood was

collected 5 h before "lights off", while in diabetic animals insulin levels may have been near maximal due to their hyperphagia and hyperglycaemia. Secondly, increased insulin resistance has been reported in streptozotocin-diabetic rats [25, 26], such that the putative insulin deficiency in this study may have been partially due to a change in insulin sensitivity. Thirdly, it has also recently been shown that streptozotocin-diabetes is associated with an increased ratio of proinsulin to insulin [27]. Consequently the levels of bio-active insulin in diabetic animals in our study may have been lower than reported. The increase in NPY mRNA in these mildly diabetic animals supports the hypothesis that NPY expression in the arcuate nucleus is responsive to small reductions in serum insulin. Because these mildly diabetic animals gained weight normally, it is unlikely that the change in arcuate NPY mRNA was in response to weight loss. However, the presence of mild hyperglycaemia does not exclude the possibility that the metabolic consequences of hypoinsulinaemia may have contributed to the effect on NPY mRNA. In particular changes in cortico- [28] and sex steroids [29] could have affected NPY expression.

In previous studies, where diabetic rats were treated with insulin, hypothalamic NPY or NPY mRNA returned to or near basal levels [15–17]. These data are consistent with a direct effect of insulin on NPY expression but do not exclude an effect secondary to the improved metabolic state and weight gain which was reported in these studies. It is also unclear how rapidly insulin affected NPY expression because of the variation in time over which insulin was administered [15–17]. In a similar experiment, we found there was a significant decrease in NPY mRNA within 20 h of insulin treatment in the rostral and mid levels of the arcuate nucleus. In the caudal level, a longer period of insulin treatment was required to achieve a decrease in NPY mRNA. The earlier effect seen in some levels may reflect heterogeneous responses within the arcuate nucleus. Such heterogeneous responses have previously been reported in fasted animals [12]. The effect of insulin treatment on NPY mRNA was unlikely to have been due to an improved metabolic state because insulin administration did not significantly improve glycaemia at least when measured, prevent weight loss or decrease food intake. Even though an indirect effect of insulin on hormone levels or fuels cannot be excluded, these results are consistent with a direct effect of insulin on NPY mRNA expression in the hypothalamus.

Although the relationship between NPY mRNA in the arcuate nucleus and amount of NPY synthesised and released in the hypothalamus is unclear, it is quite likely that there is a positive correlation. NPY release is increased in the PVN of fasted animals [13] and in isolated medio-basal hypothalami of diabetic rats [15]. In both these states, increased NPY mRNA has been found in the arcuate nucleus. Furthermore, arcuate NPY mRNA levels may be a more sensitive indicator of release than tissue levels of NPY in hypothalamic sites such as the PVN. In diabetic and fasted animals, this and previous studies [12–16], have shown that elevation of arcuate NPY mRNA occurs more rapidly and to an equal or greater extent than increases in NPY levels.

The data in this study support the hypothesis that circulating insulin can modulate NPY expression in the hypothalamus. Other supporting data include firstly, the ability of centrally administered insulin to decrease levels of immunoactive NPY in the PVN and NPY mRNA in the arcuate nucleus [20]; secondly the increase in NPY peptide and mRNA that occurs in fasted animals [8–13, 30]; thirdly the presence of high levels of insulin receptors [31] and insulin receptor mRNA [32] in the arcuate nucleus and fourthly the rapid entry of circulating insulin into the arcuate nucleus [33].

Because NPY mRNA in the arcuate nucleus responded to apparently small changes in serum insulin, it is quite possible that the physiological changes in insulin levels that occur with the diurnal variation may also regulate NPY mRNA expression and NPY release. The relatively rapid response of NPY mRNA to changes in circulating insulin found in this study make this more likely. The possibility that insulin regulates NPY expression over a much shorter period than found in this study is being investigated.

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Dr. J. Marks
Department of Clinical Endocrinology
Westmead Hospital
Westmead, NSW
Australia 2145