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# Resistin expression and regulation in mouse pituitary

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**Abstract Resistin, a new adipocytokine, is expressed in human, rat and mouse adipose tissue. Its putative role as a mediator of insulin resistance is controversial. We hypothesized that resistin, in common with leptin, has multiple roles in non-adipose tissues. Using reverse transcription polymerase chain reaction (RT-PCR) we show that the resistin gene (*Retn*) is expressed in mouse brain (hypothalamus and cortex) and pituitary gland. Immunohistochemistry revealed resistin protein in the arcuate nucleus and pituitary gland. Semi-quantitative RT-PCR analysis indicated that *Retn* mRNA is developmentally regulated in the pituitary. Expression was lowest at birth, increased abruptly between postnatal days 14 and 25 (four-fold; $P < 0.001$), and declined thereafter. This peak in pituitary *Retn* mRNA was unaffected by early weaning but was abolished by neonatal treatment with monosodium glutamate, suggesting that the basal hypothalamus regulates pituitary *Retn*. Although the role(s) of endogenous resistin in mouse brain and pituitary remains to be determined, it may be distinct from its controversial involvement in insulin resistance. Our data suggest that local resistin expression could have functional implications during prepubertal maturation of the hypothalamic–pituitary system.** 

*Key words:* Resistin; Hypothalamus; Pituitary; Reverse transcription polymerase chain reaction; Ontogeny

## 1. Introduction

Type 2 diabetes is characterized by insulin resistance in certain peripheral tissues and is often associated with obesity. However, the relationship between obesity and insulin resistance is not understood. A new adipocyte-derived circulating hormone, resistin [1], was suggested to induce insulin resistance. Resistin is part of a multigene family of cysteine-rich proteins, termed RELMs or FIZZ proteins [2]. To date three members of this family have been described, all secreted proteins with unique tissue distributions [2,3]. Resistin is expressed and secreted from mouse [1] and rat [4] fat, and differentiated 3T3L1 adipocytes [1]. Low level expression was also noted in rodent mammary gland [1]. Human resistin has been reported in fat [1,5,6] and in primary preadipocytes [7,8] though this is controversial [9,10].

Adipocyte-derived resistin was proposed to antagonize the actions of insulin [1,11], providing a putative hormonal link between obesity and diabetes. Resistin treatment impaired glucose tolerance whereas immunoneutralization improved insulin action. Serum resistin levels were down-regulated by rosiglitazone, an anti-diabetic drug which enhances insulin sensitivity. These findings were subsequently confirmed in db/db mice [12]. However, other reports challenge the role of resistin as a mediator of insulin resistance, particularly in humans. No differences in adipocyte *Retn* expression were observed between normal, insulin-resistant and type 2 diabetic patients [9], and *Retn* mRNA was undetectable in adipocytes from an insulin-resistant patient [10]. Insulin increased *Retn* gene expression in streptozotocin-diabetic mice [4] and *Retn* expression was increased by rosiglitazone in ob/ob mice and Zucker fa/fa rats [13]. A substantial reduction in *Retn* mRNA levels in 3T3L1 adipocytes treated with insulin was also observed [14].

We hypothesized that resistin could be implicated in physiological systems which are distinct from those involving adipose tissue. For example, *Retn* expression is influenced by several factors which are not restricted to control of adipose tissue, including: TNF-α [15], β-adrenergic agonists [16] and testosterone [17]. Holcomb et al. [3] suggested that the resistin-like molecule FIZZ 1 modulates the function of sympathetic neurons. Another adipocytokine, leptin, is also made in brain and pituitary [18–20]. Since adipose *Retn* expression is regulated via the peroxisome proliferator-activated receptor gamma, which is also localized to the brain [21], the present study was undertaken to determine whether resistin was expressed in the developing brain and pituitary of the mouse.

## 2. Methods

### 2.1. Mice

Adult CD1 and C57BL 6 (female; 40 days old) mice were obtained from Charles River Breeding Farms (Quebec, Canada), maintained under a photoperiod of 14 h light:10 h darkness (lights on: 0700 h) and given free access to Purina Rat Chow and drinking water. The experimental protocol was reviewed and approved by the Dalhousie University Committee on Laboratory Animals. Mice were killed by decapitation and samples of frontal cerebral cortex, basal hypothalamus and visceral/gonadal fat were dissected and frozen in liquid nitrogen. The pituitary gland was removed intact (anterior plus posterior). Each *n* value represents tissue pooled from three to four mice.

For the developmental study, female CD1 mice were received with their mothers, on postnatal day (PD) 2. Pups were weighed and killed by decapitation on PDs 3, 7, 14, 18, 21, 25, 28 and 40. Intact pituitary glands and hypothalami were collected for analysis of resistin mRNA levels. Newborn CD1 pups were also treated with monosodium glutamate (MSG; 4 mg/g; s.c.; Sigma) on PDs 3 and 4. Control mice received saline. Pups remained with their mothers and were killed by decapitation on PD 21. Intact pituitaries and visceral fat were collected for reverse transcription polymerase chain reaction (RT-PCR)

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analysis. Each *n* value represents pooled tissue from a minimum of four mice. Data were derived from two independent experiments.

*2.2. RNA isolation and semi-quantitative RT-PCR analysis*

Total RNA was isolated from brain and pituitary using the RNeasy mini kit (Qiagen; Mississauga, ON) and from adipose tissue using Trizol reagent (Gibco). The RNA was DNase treated using the RNase-free DNase kit (Qiagen). RNA (1.75 μg) was denatured at 65°C for 5 min and reverse transcribed in a final volume of 30 μl using Omniscript reverse transcriptase (Qiagen) at 37°C for 1 h. PCR amplification was performed using two alternate sets of intron-spanning (278 bp, R1, and 330 bp, R2) resistin primers and HotStar*Taq* DNA polymerase (Qiagen). The sequences of the primers are as follows: R1 forward, 5′-TTCCTTGTCCCTGAACTGCT-3′, reverse, 5′-TGCTGTCCAGTCTATCCTTG-3′ and R2 forward, 5′-GCTGTGGGACAGGAGCTAATA-3′, reverse, 5′-GTCCCACGAGCCACAGGCAGA-3′. The PCR reaction was performed as described previously [20]. Reactions were normalized by evaluating the level of amplification of the 18S transcript using commercial primers (Classic 18S primers; Ambion, Austin, TX). For semi-quantitative analysis, each sample was amplified within the exponential phase of the PCR (pituitary: resistin, 30 PCR cycles; 18S 20–21 cycles). For each target no amplification product was detected in the absence of reverse transcriptase. PCR-amplified DNA was electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining. Product yield was determined using NIH Image (v.1.60) software. Data were expressed as a ratio relative to 18S in arbitrary units.

*2.3. Cloning and sequencing*

Total RNA from hypothalamus and gonadal fat was reverse transcribed and PCR-amplified as described above. Portions of the resulting PCR reaction were cloned into pGEM-T Easy (Promega, Madison, WI) and transformed into *Escherichia coli*. Individual transformants were sent for sequence analysis to the Dalhousie University–NRC Institute for Marine Biosciences Joint Laboratory (Halifax, NS).

*2.4. Saline perfusion washout of blood cells*

Male mice (approximately PD 40) were anesthetized with somnotol (65 mg/kg; i.p.) and perfused with sterile saline (room temperature; 40 ml per mouse) to remove blood contamination from the brain and pituitary. A second group was anesthetized but not perfused. Samples of hypothalamus and pituitary glands were obtained as described (see Section 2.1).

*2.5. Immunohistochemistry*

Resistin protein was detected in fixed tissue from brain and pituitary gland (4% paraformaldehyde) [22] using a rabbit polyclonal antiserum (Phoenix Pharmaceuticals; # H-028-40; 1:12 000 (brain) or 1:1000 (pituitary).

*2.6. Statistical analysis*

Data were analyzed by Student's *t*-test or analysis of variance with Newman–Keuls post hoc test and are reported as mean ± S.E.M.

## 3. Results

*3.1. Retn mRNA is expressed in mouse brain and pituitary*

*Retn* expression in mouse brain and pituitary was evaluated in adult C57BL 6 and CD1 mice using RT-PCR analysis with two alternate sets of resistin primers designed to span 278 and 330 bp of the cDNA. Products of the expected sizes were reproducibly observed in fat, hypothalamus, cortex and whole pituitary gland using both sets of primers in both strains of mice (Fig. 1A). *Retn* expression was readily detectable even after 30 cycles. No PCR product was detected using either set of PCR primers in the negative control or in the absence of reverse transcriptase. The 278 bp and 330 bp PCR amplicons were cloned and sequence analysis indicated 100% homology with the corresponding region of mouse resistin cDNA [1]. Since low level expression of resistin has been reported in

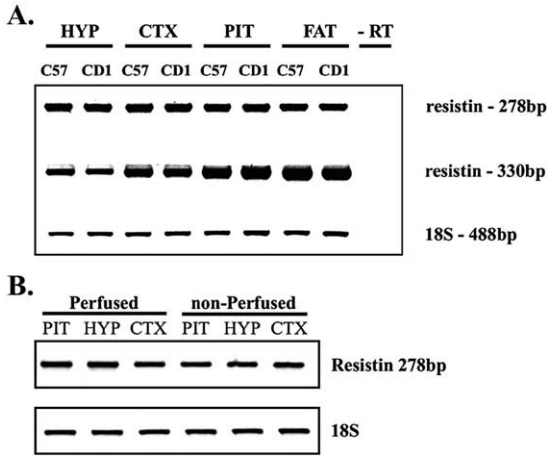


Fig. 1. A: *Retn* expression in mouse brain and pituitary. RT-PCR analysis of RNA isolated from the hypothalamus (HYP), cortex (CTX), pituitary (PIT) and visceral fat of adult female C57BL6 and CD1 mice using two alternate sets of intron-spanning *Retn* primers. Sequence analysis of the 278 bp and 330 bp amplicons revealed 100% homology with the corresponding region of murine *Retn* cDNA. B: RT-PCR analysis of *Retn* expression (278 bp) in the brain and pituitary of adult male CD1 mice which had been perfused with sterile saline (40 ml) and from non-perfused control mice.

human monocytes [9,10], we compared *Retn* mRNA levels in saline-perfused and non-perfused tissues. Our results indicated that resistin expression was identical in both perfused and non-perfused mouse brain and pituitary (Fig. 1B). *Retn* expression in microdissected arcuate nuclei (ARCs) was compared to expression in the remainder of the hypothalamus using semi-quantitative RT-PCR analysis (278 bp primers). Resistin expression was significantly higher (∼three-fold; $n=4$; $P<0.005$) within the ARC compared to the remainder of the hypothalamus (data not shown), consistent with the immunolocalization of resistin protein (see Fig. 2).

*3.2. Immunohistochemical localization of resistin*

Resistin immunoreactivity was localized to scattered cell bodies in the ARC, with additional small amounts present in the ventrolateral ventromedial hypothalamus and in the dorsal periventricular area (Fig. 2A,C). Resistin immunoreactivity was also present in anterior and intermediate lobes of the pituitary, but little staining was seen in posterior lobe (Fig. 2D). Staining was abolished by preadsorption of the antiserum with the immunizing peptide or by removal of the primary antiserum (Fig. 2B,E).

*3.3. Developmental regulation of resistin mRNA in mouse pituitary*

Developmental changes in resistin mRNA levels were evaluated between PDs 3 and 40 in female CD1 mouse pituitary and hypothalamus using semi-quantitative RT-PCR analysis. For each tissue, amplification was performed within the exponential phase of the PCR reaction (pituitary 31 cycles, hypothalamus 32 cycles). *Retn* mRNA was detected in the hypothalamus and pituitary throughout neonatal development (PDs 3–40), though age-dependent changes in *Retn* mRNA levels were noted in the pituitary, where a peak in *Retn* expression was observed at PD 21 (non-weaned; Fig. 3A; ∼four-fold between PDs 14 and 21; $n=7$; $P<0.001$). A

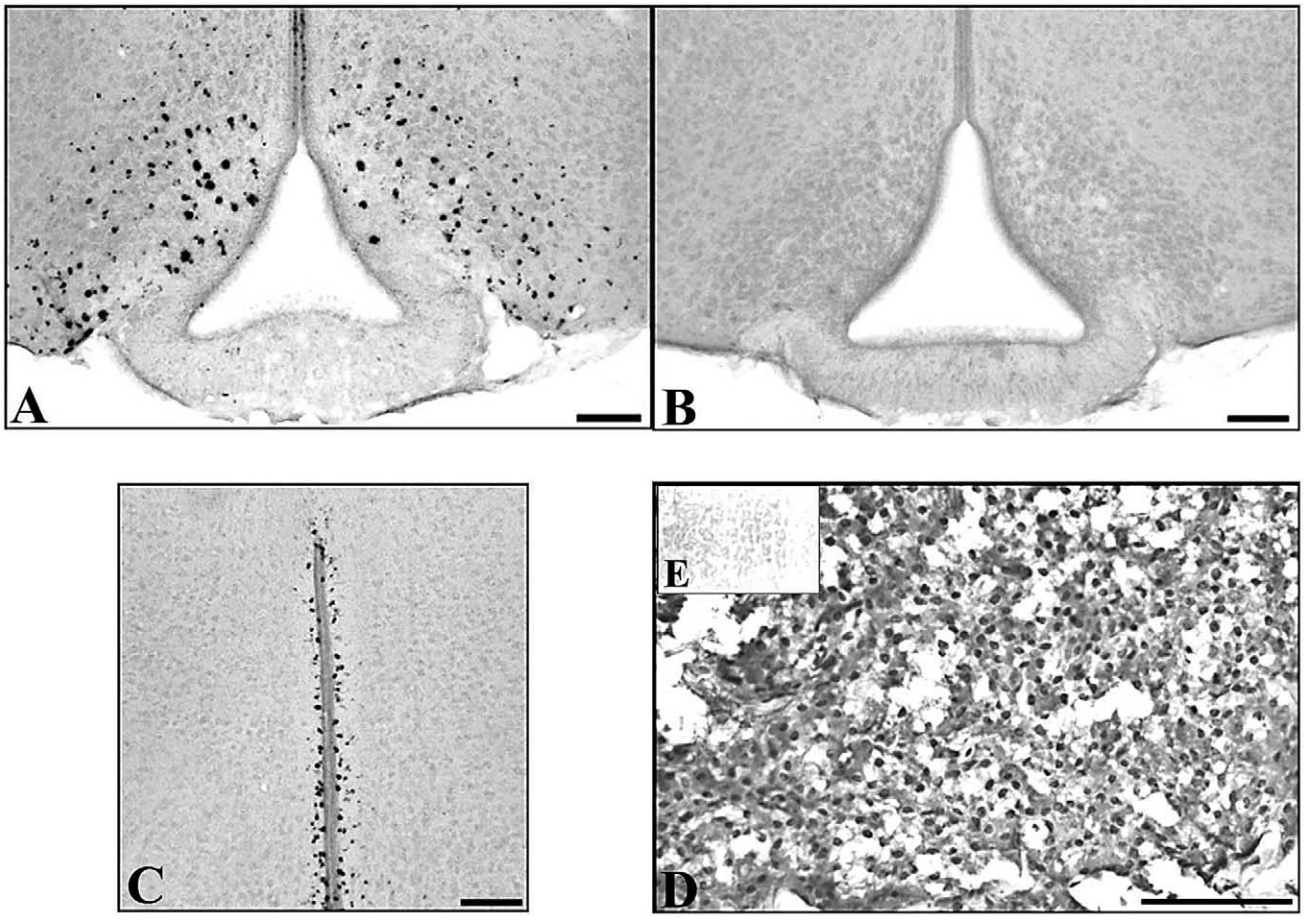


Fig. 2. Immunolocalization of resistin in mouse brain and pituitary. Representative images of resistin immunoreactivity in coronal sections of the ARC (A), dorsal periventricular region of the hypothalamus (C) and anterior pituitary (D) obtained from male CD1 mice perfusion-fixed with 4% paraformaldehyde. Note that immunoreactivity was abolished following removal of the primary antiserum (B,E). Scale bars are 100 μm.

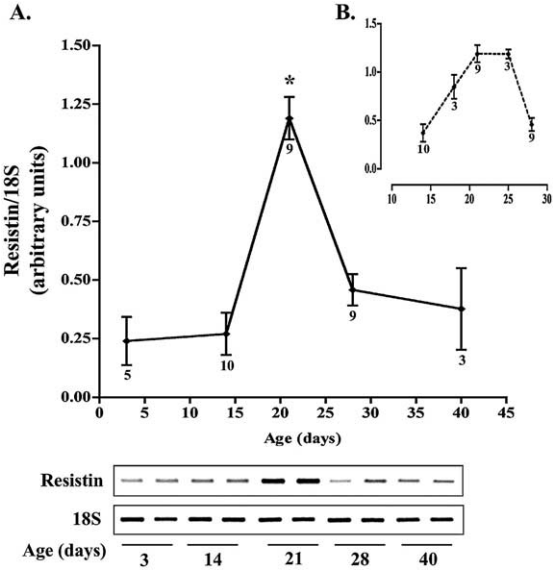


Fig. 3. Developmental regulation of *Retn* expression in mouse pituitary. Intact pituitaries were collected from CD1 mice on PDs 3, 14, 21, 28 and 40 (A) or PDs 14, 18, 21, 25, and 28 (B) for RNA isolation and semi-quantitative RT-PCR analysis of *Retn* expression. Representative ethidium bromide-stained gels are shown. Cumulative results (mean ± S.E.M.) are shown. Numbers below standard error bars represent *n* values. $^{*}P < 0.001$; PD 21 vs PDs 3, 14, 28, 40.

closer evaluation of the time interval surrounding this peak in resistin expression revealed a gradual increase and decline between PDs 14 and 28 with maximal levels between PDs 21 and 25 (Fig. 3B). We compared resistin expression in mice which had been weaned for 6 and 24 h and in non-weaned mice (PD 21). The increase in resistin expression at PD 21 was unaffected by weaning. By contrast, in the hypothalamus, although resistin was expressed throughout postnatal development (PDs 3–40) no significant age-related differences were observed (data not shown).

To determine whether pituitary *Retn* expression was dependent on an intact hypothalamus, the ARC was neurotoxically lesioned with neonatal MSG treatment [22,23]. Semi-quantitative analysis of *Retn* mRNA in pituitaries of PD 21 male mice revealed a marked attenuation of resistin expression (Fig. 4). In preliminary experiments, a similar effect on pituitary *Retn* expression was observed in female CD1 mice. *Retn* expression in visceral fat (PD 21) was not affected by neonatal MSG treatment (data not shown).

## 4. Discussion

This study represents the first demonstration of resistin mRNA expression in mouse brain and pituitary. In addition to adipocytes, *Retn* mRNA was reported in rodent mammary gland [1]. Holcomb et al. [3] detected *Retn* mRNA in several

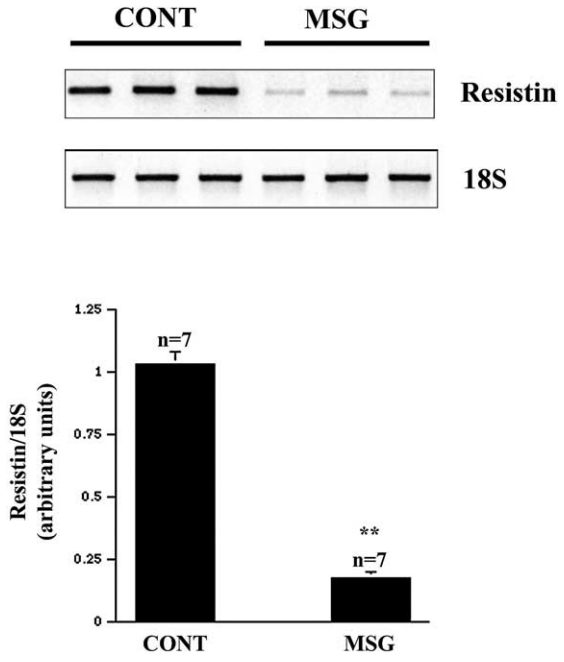


Fig. 4. Effects of neonatal MSG treatment on pituitary *Retn* expression. Intact pituitaries were collected from male CD1 mice on PD 21 which had either been treated with MSG (PDs 3 and 4) or saline (control) and subjected to semi-quantitative RT-PCR analysis of resistin expression. Cumulative results (mean ± S.E.M.) are shown in a histogram. Each *n* value (shown above bars) represents tissue pooled from a minimum of four mice. $^{**}P < 0.005$; Student's *t*-test.

organs, though these data were not reported. Low level expression was also found in human monocytes [9,10], but in our hands resistin was expressed equally in saline-perfused and non-perfused mouse pituitary and hypothalamus, suggesting that monocytes are not a significant source of resistin in mouse pituitary and brain. Our results indicated that *Retn* mRNA was localized to the ARC of the hypothalamus, and immunohistochemistry confirmed the presence of resistin protein. At present the phenotype of these resistin-positive cells is unknown, though the periventricular cells could be tanycytes or astrocytes based on their appearance (Fig. 2). Positive staining was also seen in the anterior and intermediate lobes of the pituitary, confirming the strong *Retn* mRNA expression. The role of resistin in the hypothalamo–pituitary system is unclear, but peripheral *Retn* mRNA and serum levels are regulated by fasting/refeeding [1,4], insulin [1,4,13,14], testosterone [17] and in genetic models of obesity [1,13]. In future experiments it will be important to determine the effects of each of these factors on brain and pituitary resistin expression.

The significance of the developmental rise in *Retn* mRNA levels between PDs 14 and 21 in mouse pituitary is unknown. The peak in pituitary resistin expression occurs over a 2 week period, just prior to puberty, between PDs 14 and 28, with maximal levels between PDs 21 and 25. By comparison, hypothalamic levels remained constant throughout development. The increase in *Retn* expression appeared to be coincident with the time of weaning. However, our results do not support a direct effect of weaning since a similar increase was observed in both weaned and non-weaned mice. In further studies we determined whether pituitary *Retn* mRNA was dependent upon an intact hypothalamus. Treatment of neonatal rodents with MSG is an accepted approach to selectively ablate the ARC of the hypothalamus [24] and to influence developmental changes in the pituitary gland [25]. Our data show that pituitary *Retn* expression is dependent upon an intact hypothalamus, though it remains to be determined which pituitary cell type(s) express resistin protein. It may also be significant that MSG-treated mice become obese as adults and show hyperglycemia and hyperinsulinemia, although surprisingly resistin expression in visceral fat was unaffected [26].

We have recently reported developmental regulation of leptin mRNA in rat pituitary, where expression was maximal during PDs 7–14 and fell sharply by PD 22. The role of endogenous leptin in the pituitary has not been determined, but our data, together with those for resistin, suggest that local adipocytokine expression may have functional significance in the development of the brain–pituitary system. As with leptin, additional unexpected roles for resistin are possible. For example, in addition to its well characterized role as a satiety factor, leptin has been reported to be important for neuronal maturation [20,27,28]. Considering the strong association of obesity and diabetes, it will be important to determine whether resistin and leptin co-localize in the brain and pituitary and to extend these studies to other species.

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## References

[1] Steppan, C.M., Bailey, S.T., Bhat, S., Brown, E.J., Banerjee, R.R., Wright, C.M., Patel, H.R., Ahima, R.S. and Lazar, M.A. (2001) Nature 409, 307–312.
[2] Steppan, C.M., Brown, E.J., Wright, C.M., Bhat, S., Banerjee, R.R., Dai, C.Y., Enders, G.H., Silberg, D.G., Wen, X., Wu, G.D. and Lazar, M.A. (2001) Proc. Natl. Acad. Sci. USA 98, 502–506.
[3] Holcomb, I.N., Kabakoff, R.C., Chan, B., Baker, T.W., Gurney, A., Henzel, W., Nelson, C., Lowman, H.B., Wright, B.D., Skelton, N.J., Frantz, G.D., Tumas, D.B., Peale Jr., F.V., Shelton, D.L. and Hebert, C.C. (2000) EMBO J. 19, 4046–4055.
[4] Kim, K.H., Lee, K., Moon, Y.S. and Sul, H.S. (2001) J. Biol. Chem. 276, 11252–11256.
[5] McTernan, C.L., McTernan, P.G., Harte, A.L., Levick, P.L., Barnett, A.H. and Kumar, S. (2002) Lancet 359, 46–47.
[6] McTernan, P.G., McTernan, C.L., Chetty, R., Jenner, K., Fisher, F.M., Lauer, M.N., Crocker, J., Barnett, A.H. and Kumar, S. (2002) J. Clin. Endocrinol. Metab. 87, 2407–2410.
[7] Sharma, A.M., Engeli, S., Janke, J., Gorzelniak, K. and Luft, F.C. (2001) Obesity Research 9, Suppl. 3, PE 25.
[8] Janke, J., Engeli, S., Gorzelniak, K., Luft, F.C. and Sharma, A.M. (2002) Obes. Res. 10, 1–5.
[9] Nagaev, I. and Smith, U. (2001) Biochem. Biophys. Res. Commun. 285, 561–564.
[10] Savage, D.B., Sewter, C.P., Klenk, E.S., Segal, D.G., Vidal-Puig, A., Considine, R.V. and O'Rahilly, S. (2001) Diabetes 50, 2199–2202.
[11] Steppan, C.M. and Lazar, M.A. (2002) Trends Endocrinol. Metab. 13, 18–23.
[12] Moore, G.B., Chapman, H., Holder, J.C., Lister, C.A., Piercy, V., Smith, S.A. and Clapham, J.C. (2001) Biochem. Biophys. Res. Commun. 286, 735–741.
[13] Way, J.M., Gorgun, C.Z., Tong, Q., Uysal, K.T., Brown, K.K., Harrington, W.W., Oliver Jr., W.R., Willson, T.M., Kliewer, S.A. and Hotamisligil, G.S. (2001) J. Biol. Chem. 276, 25651–25653.
[14] Haugen, F., Jorgensen, A., Drevon, C.A. and Trayhurn, P. (2001) FEBS Lett. 507, 105–108.

[15] Fasshauer, M., Klein, J., Neumann, S., Eszlinger, M. and Paschke, R. (2001) Biochem. Biophys. Res. Commun. 288, 1027–1031.
[16] Fasshauer, M., Klein, J., Neumann, S., Eszlinger, M. and Paschke, R. (2001) FEBS Lett. 500, 60–63.
[17] Ling, C., Kindblom, J., Wennbo, H. and Billig, H. (2001) FEBS Lett. 507, 147–150.
[18] Morash, B., Li, A., Murphy, P., Wilkinson, M. and Ur, E. (1999) Endocrinology 140, 5995–5997.
[19] Wilkinson, M., Morash, B. and Ur, E. (2000) Front. Horm. Res. 26, 106–125.
[20] Morash, B., Willkinson, D., Murphy, P., Ur, E. and Wilkinson, M. (2001) Mol. Cell. Endocrinol. 185, 151–159.
[21] Rohn, T.T., Wong, S.M., Cotman, C.W. and Cribbs, D.H. (2001) NeuroReport 12, 839–843.
[22] Natarajan, M. and Wilkinson, M. (1997) Brain Res. Dev. Brain Res. 102, 97–104.
[23] Seress, L. (1982) Neuroscience 7, 2207–2216.
[24] Olney, J.W. and Price, M.T. (1978) in: Kainic Acid as a Tool in Neurobiology (McGeer, E.G., Ed.), pp. 239–263, Raven Press, New York.
[25] Sasaki, F., Kawai, T. and Ohta, M. (1994) Anat. Rec. 240, 255–260.
[26] Papa, P.C., Seraphim, P.M. and Machado, U.F. (1997) Int. J. Obes. Relat. Metab. Disord. 21, 1065–1070.
[27] Ahima, R.S., Bjorbaek, C., Osei, S. and Flier, J.S. (1999) Endocrinology 140, 2755–2762.
[28] Steppan, C.M. and Swick, A.G. (1999) Biochem. Biophys. Res. Commun. 256, 600–602.