Review Article

Neuropeptide Y in the noradrenergic neurons induces the development of cardiometabolic diseases in a transgenic mouse model

Suvi T. Ruohonen[1,2], Ullamari Pesonen[1], Eriika Savontaus[1,2,3]

[1]Department of Pharmacology, Drug Development and Therapeutics and, [2]Turku Center for Disease Modeling, University of Turku, [3]Unit of Clinical Pharmacology, Turku University Hospital, Turku, Finland

ABSTRACT

Neuropeptide Y (NPY) is a neuropeptide widely expressed in the brain and a peptide transmitter of sympathetic nervous system (SNS) co-released with noradrenaline (NA) in prolonged stress. Association of a gain-of-function polymorphism in the human NPY gene with dyslipideamia, diabetes and vascular diseases suggests that increased NPY plays a role in the pathogenesis of the metabolic syndrome in humans. In the hypothalamus, NPY plays an established role in the regulation of body energy homeostasis. However, the effects of NPY elsewhere in the brain and in the SNS are less explored. In order to understand the role of NPY co-expressed with NA in the sympathetic nerves and brain noradrenergic neurons, a novel mouse model overexpressing NPY in noradrenergic neurons was generated. The mouse displays metabolic defects such as increased adiposity, hepatosteatosis, and impaired glucose tolerance as well as stress-related hypertension and increased susceptibility to vascular wall hypertrophy. The mouse phenotype closely reflects the findings of the several association studies with human NPY gene polymorphisms, and fits with the previous work on the effects of stress-induced NPY release on metabolism and vasculature. Thus, in addition of promoting feeding and obesity in the hypothalamus, NPY expressed in the noradrenergic neurons in the brain and in the SNS induces the development of cardiometabolic diseases.

Key words: Adipose tissue, atherosclerosis, diabetes, hypertension, neuropeptide Y, noradrenaline, obesity, sympathetic nervous system

Introduction

Neuropeptide Y (NPY) is one of the most common peptides in the central nervous system (CNS) and an abundant neurotransmitter in the peripheral sympathetic nervous system (SNS). NPY has been linked to several disorders associated with the metabolic syndrome and vascular diseases in humans carrying a gain-of-function polymorphism[1] and in animal models. Although it is well-established that NPY plays a key role in the hypothalamic control of body energy balance by promoting feeding and lipid storage in white adipose tissue (WAT), other mechanisms are likely to take part in inducing the development of cardiometabolic diseases. This review summarizes the work on a mouse model overexpressing NPY in noradrenergic neurons that displays obesity, impaired glucose tolerance and susceptibility to vascular disease. The role of increased NPY in the CNS and the SNS, and the relevance to human disease are discussed.

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Neuropeptide Y

NPY is a 36 amino acids long tyrosine-rich peptide structurally related to pancreatic polypeptide (PP) and peptide YY (PYY) that all mediate their effects by Y-receptors, a family of G-protein coupled receptors

Corresponding Author: Dr. Eriika Savontaus, Department of Pharmacology, Drug Development and Therapeutics, University of Turku, Finland. E-mail: eriika.savontaus@utu.fi

expressed ubiquitously in the brain and in the peripheral tissues.[2] The highest concentrations of NPY in mammals are found in the hypothalamic nuclei central to the control of energy homeostasis and the neuroendocrine system: The paraventricular hypothalamic nucleus (PVN) and the hypothalamic arcuate nucleus (ARC). The hypothalamic NPYergic neurons respond to changes in peripheral hormones like leptin, and are activated in states of negative energy balance such as starvation, which leads to increased feeding, decreased energy expenditure and increased storage of energy in fat. In addition, NPY is co-localized with noradrenaline (NA) in the brain.[3-6] The main noradrenergic sites containing NPY are located in the brainstem. These include the locus coeruleus (LC) and A1/C1 cell groups in the ventrolateral medulla oblongata,[3,7] which play important roles in controlling arousal and autonomic nervous system. Similar to the third cerebral ventricle in the hypothalamus, administration of NPY to the fourth ventricle in the brainstem increases food intake.[8,9] This effect seems to be mediated in part by NPY containing noradrenergic neuron projections to the PVN[9-11]

In the periphery, NPY is mainly expressed in NA-producing neurons in the SNS, which governs the emergency reaction, also referred to as the *fight-or-flight* reaction, to prepare the body to an alert mode ready to utilize its energy stores. NPY co-stored with NA is also released upon sympathetic activation, but unlike NA, NPY is preferentially released by intense and prolonged sympathetic activation being a marker of a more severe stress.[12] Sympathetic neurons containing NPY innervate several tissues including cerebral vasculature,[13,14] heart,[15] thyroid gland,[16] respiratory tract,[17] gut,[18,19] pancreas,[18] liver,[20,21] fat,[22] brown fat[23] and eye.[24] NPY is also found in adrenal glands, where it is produced by adrenaline- and NA-producing chromaffin cells.[25,26] NPY in plasma mainly derives by spillover from sympathetic nerves and adrenal glands, strong sympathetic activation being required to release enough NPY to raise the circulating levels.[27,28] In the sympathoadrenal system, NPY is thought to modulate the effects of NA for instance prolong and enhance vasoconstriction. On the other hand, the effects of NA and NPY can be opposite like in the control of lipolysis. NPY may also regulate the synthesis and release of NA.[29-31]

Mouse Model of Neuropeptide Y Overexpression in Noradrenergic Neurons

In order to understand the role of NPY co-expressed with NA in the sympathetic nerves and brain noradrenergic neurons, a novel mouse model overexpressing NPY in noradrenergic neurons of the brain and periphery was generated.[32] In this model, NPY is overexpressed under the human dopamine-beta-hydroxylase (DBH) promoter (OE-NPY^{DBH} mouse). The transgene construct included a reporter gene *LacZ* encoding β-galactosidase, which allowed verifying that the promoter drove the expression of the transgene to correct sites. The transgenic mice were initially created in FVB/n background, but detailed phenotyping was performed after backcrossing the mice for at least six generations to C57BL/6 N, a strain susceptible to obesity and metabolic disturbances. The phenotyping studies were performed on mice heterozygous for the transgene comparing to wildtype littermates.

After verifying that the transgene was intensely expressed in the adrenal glands and in the noradrenergic nuclei LC and A1/C1 in the brainstem, the overproduction of NPY was quantitated in tissue homogenates and plasma with radioimmunoassay (RIA). At baseline, the OE-NPY^{DBH} mice had a significantly higher NPY concentration in the adrenals (1.3-fold) and in the brainstem (1.8-fold) compared with wildtype mice. Although it has previously been shown that substantial amount of NPY in the PVN and some other hypothalamic nuclei orginates from the brainstem,[11] no differences between genotypes were detected in NPY concentration in the hypothalamus. Plasma NPY levels did not differ between the genotypes at baseline, but were increased after acute sympatho-adrenal stressors restraining and cold (4°C). The response to restrained stress was increased in OE-NPY^{DBH} female mice compared to female wildtype mice, but was not different between genotypes in males or after cold stress. The more pronounced increase in plasma NPY levels in female OE-NPY^{DBH} after sympathetic activation suggested a sex-related difference in the effect of NPY on adrenal excitability, which was also evident in female $Y_1^{-/-}$ mice showing enhanced catecholamine release after stress.[33] In conclusion, NPY levels were increased in the adrenals and the brainstem in the OE-NPY^{DBH} mice, and the stress-induced increase in the plasma was enhanced in female OE-NPY^{DBH} mice.

Adiposity and Energy Homeostasis in OE-NPY^{DBH} Mice

Centrally infused NPY induces obesity in long-term[34,35] and increased hypothalamic NPY concentrations seem to be a major component at the origin of obesity in several obese animal models.[36-39] Increased NPY signaling plays also a role in hyperphagia and obesity induced by disruption of catecholaminergic projections from brainstem to the hypothalamus.[40] However, the role of NPY in the circulation and SNS in the regulation of energy homeostasis has not been

widely studied, although NPY and its receptors are located in key peripheral tissues, such as WAT, liver, and pancreas. With OE-NPY^{DBH} mice, body weights were recorded from male and female mice at different ages, and no difference between the genotypes was observed.[32] No difference in the amount of food consumed between the wildtype and transgenic mice was observed either. Spontaneous locomotor activity and rectal body temperatures were also similar between the genotypes. Despite these findings, OE-NPY^{DBH} mice had significantly more WAT enriched in smaller adipocytes than wildtype controls, and increased deposition of triglycerides in the liver.[32] The difference in adiposity between genotypes was evident already in young adults and continued to increase by late adulthood. The difference was larger in males than in females when the mice were fed with regular chow diet. In contrast, when fed with an energy-dense Western-type diet, female OE-NPY^{DBH} mice gained more weight compared with their wildtype littermates (40% vs. 26% of initial weight).[41] In line with this, female transgenic mice also had larger WAT and brown adipose tissue (BAT) mass. In males, prominent weight gain was observed in both genotypes (55% of initial weight), but no difference in the weight gain or WAT mass was observed between OE-NPY^{DBH} and wildtype mice.

Obesity is characterized by an increase in WAT mass, which occurs via increase in cell size (hypertrophy) and/or increase in cell number (hyperplasia). Hypothalamic NPY has been linked to fat cell hypertrophy by stimulating lipoprotein lipase activity in WAT.[42,43] Fat cells from the transgenic OE-NPY^{DBH} mice were significantly smaller and due to the heavier WAT weight probably more numerous. Thus, OE-NPY^{DBH} mice exhibit hyperplastic adipose tissue. The adipose tissue phenotype of OE-NPY^{DBH} mice fits with the effects of stress-induced NPY release on energy homeostasis.[44] Kuo *et al.* demonstrated that NPY exerts obesity by local effects (increased adipogenesis and angiogenesis) in the WAT via Y_2 receptors without changes in food intake or body weight. Fat accumulation was attenuated by local Y_2 receptor antagonist administration and fat-targeted Y_2-gene knockdown procedure.[44] The peripheral action of NPY can also involve the tonic regulation of fat oxidation and energy expenditure as shown in recent studies using conditional peripheral Y_1 and Y_2 null mice.[45,46] Furthermore, SNS NPY could induce lipogenesis by inhibiting lipolysis via adipocyte-located Y_1-receptors.[47] Hence, all these data combined show that NPY can induce obesity by extrahypothalamic pathways influencing generation, differentiation and function of adipocytes and use of lipids as energy source rather than by influencing appetite via hypothalamic sites or brainstem – hypothalamus projections.

Non-Shivering Thermogenesis in OE-NPY^{DBH} Mice

BAT is the main site of thermogenesis in rodents and plays a role in the regulation of body weight. Inactive BAT contributes to weight gain in many animal models of obesity. The main physiological regulator of BAT non-shivering thermogenesis is NA released by sympathetic neurons densely innervating the BAT. Binding of [^{3}H] GDP to BAT mitochondria is an estimate measure of non-shivering thermogenesis and thus sympathetic activity.[48-50] Hypothalamic NPY has been shown to decrease SNS activity assessed by reductions in BAT thermogenesis in rats.[42] Surprisingly, [^{3}H]GDP binding was significantly increased in OE-NPY^{DBH} BAT mitochondria compared with wildtype mice, and the difference was maintained after cold stress.[51] Thus, it can be concluded that defective BAT thermogenesis does not contribute to the development of obesity in OE-NPY^{DBH} mice.

Glucose Homeostasis in OE-NPY^{DBH} Mice

Impaired glucose tolerance (IGT) and insulin resistance accompanying obesity are an integral part of the metabolic syndrome. In OE-NPY^{DBH} mice, IGT was evident in six-month-old male mice, but glucose tolerance was normal in younger male and in all female mice.[32] Six-month-old males had also increased plasma insulin in comparison with wildtype males, and the insulin values correlated positively with body weights in both genotypes.[32] Female OE-NPY^{DBH} mice also displayed increased adiposity, but less fat accumulation over age than males, which is most likely the reason why glucose metabolism remained normal in chow-fed OE-NPY^{DBH} females. However, under high caloric conditions with the Western type diet, fat accumulated to a high degree and glucose tolerance was significantly impaired in female OE-NPY^{DBH} mice compared with wildtype controls.[41] Thus, a certain degree of increased adiposity i.e. increases in WAT mass and hepatic steatosis is needed before IGT occurs in OE-NPY^{DBH} mice. In line with our data, Kuo *et al.* demonstrated that increased adiposity induced by chronic mild stress and high-energy diet was accompanied with IGT.[44] It is unclear if IGT was entirely due to increased WAT mass and ectopic lipid accumulation or if increased NPY had also direct effects on glucose metabolism in OE-NPY^{DBH} mice. Central NPY has been shown to increase insulin secretion,[52,53] but peripheral NPY inhibits insulin release from β-cells.[54] Thus, it is possible that inhibition of insulin by NPY contributed to slower clearance of glucose during glucose tolerance test.

Cardiovascular Function in OE-NPYDBH Mice

Hypertension often accompanies the metabolic diseases and importantly contributes to the development of vascular diseases. Although central NPY administration inhibits sympathetic activity and decreases blood pressure,[55] peripherally NPY acts as a vasoconstrictor and increases blood pressure via stimulation of Y_1- receptors on vascular smooth muscle cells (VSMC).[56] Increased NPY levels and responses are central to the development of hypertension in animal models of hypertension.[57,58] Blood pressure and heart rate were measured in male OE-NPY^{DBH} mice using a radiotelemetric method. Interestingly, OE-NPY^{DBH} mice showed significantly increased nighttime mean arterial pressure in comparison with wildtype littermates during the stress of recovering from the surgical implantation of the transmitters.[51] After the recovery at baseline or during the light phase of the day, no difference between the genotypes was observed. It is also noteworthy that no difference in heart rate was observed, which may be due to the enhanced blood pressure-induced baroreceptor reflex, which returns heart rate to normal. These results correlate well with results from the NPY overexpressing rat model, in which higher tail blood pressure responses were obtained with a stressful tail-cuff method, but the outcome at baseline was different with long-term radiotelemetric method.[59,60] Thus, these data together suggest that NPY does not significantly contribute to maintaining resting blood pressure, but increased NPY in the sympathetic neurons induces hypertension in association with stress.

NPY has been shown to increase DNA synthesis and cell proliferation rate in rat, porcine and human aortic VSMC lines,[61-63] and thus, increased production and release of NPY may be in part responsible for these vascular diseases. Femoral artery angioplasty, a model for studying arterial restenosis, was performed on OE-NPY^{DBH} mice.[64] The neointima formation calculated as a lesion area inside the internal elastic lamina was more pronounced in OE-NPY^{DBH} mice than in wildtype control mice.[65] The transgenic mice also appeared to experience more severe growth of the medial area after the injury than the wildtype mice. In OE-NPY^{DBH} mice, the injury significantly increased the arterial medial area from the uninjured contralateral side, but no such changes were observed in wildtype controls. Current results are in line with previous work on the effects of locally administered NPY pellets and stress-induced increase in NPY in the rat endovascular injury model, where increased formation of neointimal and medial areas was observed.[66,67] Our results suggest that increased NPY in sympathetic nerves and brain noradrenergic neurons leads to increased susceptibility to arterial thickening after vascular injury possibly due to increased VSMC proliferation activity. Therefore, NPY may increase the risk of vascular disease by several mechanisms: By directly acting on the vasculature, by increasing blood pressure, and by inducing obesity and disturbances in glucose and lipid metabolism [Figure 1].

OE-NPY^{DBH} Mice and Other Regulatory Mechanisms

Although the metabolic and cardiovascular changes in OE-NPY^{DBH} mice could be explained by direct effects of excess NPY released from the SNS, it is possible that they were generated by changes in other regulatory mechanisms induced by increased NPY in the CNS. The changes in the most likely candidates, catecholamines and the hypothalamus-pituitary-adrenal (HPA)-axis, were studied in OE-NPY^{DBH} mice.

Under anesthesia OE-NPY^{DBH} mice had significantly higher levels of adrenaline in plasma and a trend to increased NA levels whereas in adrenal glands the levels were reduced in comparison with wildtype tissues suggesting increased release of catecholamines.[51] These results are in line with previous studies showing that NPY enhances the secretion of catecholamines from human, mouse and rat adrenal chromaffin cells *in vitro*.[29-31] The secretion is controlled via Y_1 receptors, because the effect is abolished in Y_1 null mice.[30] The increased plasma levels of catecholamines in OE-NPY^{DBH} mice fit well with the increased BAT activity

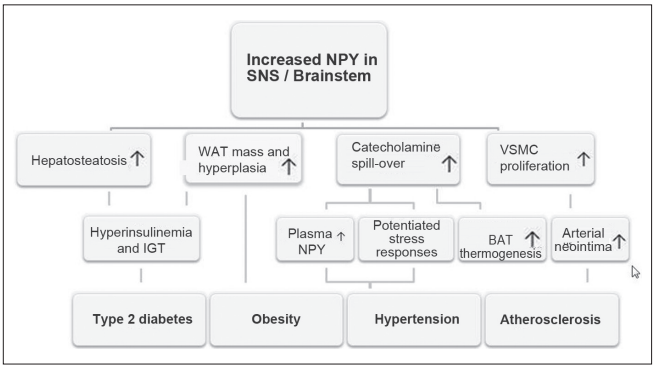

Figure 1: Putative mechanisms of neuropeptide Y (NPY) in the development of the metabolic syndrome. NPY levels are chronically increased in the brainstem and in the sympathoadrenal system in the OE-NPY^{DBH} mice. In humans, increased NPY is evident in subjects carrying the gain-of-function polymorphism or during prolonged psychosocial stress. Increased NPY enhances the development of medical disorders associated with the metabolic syndrome by inducing obesity via increased adipogenesis and lipogenesis. This is accompanied by hepatic steatosis. Obesity and fatty liver will cause insulin resistance as evidenced by impaired glucose tolerance (IGT) and hyperinsulinemia, which may eventually lead to type 2 diabetes. Hypertension is a result of increased NPY and enhanced release of catecholamines. All these disturbances together with a direct effect of NPY on vascular wall will increase the risk of atherosclerosis. VSMC = vascular smooth muscle cell; ↑ = increased

observed in males [Figure 1]. It could also contribute to increased blood pressure. However, it is unlikely that increased catecholamines played a role in increased adiposity.

Glucocorticoids have potent effects on metabolism and excess levels cause obesity and disturbances in glucose metabolism. NPY stimulates glucocorticoid release via stimulation of corticotropin-releasing hormone (CRH) in the hypothalamus.[68] Glucocorticoids and CRH are key regulators of stress responses, and acute restraint and cold stresses stimulated the HPA-axis and increased the secretion of corticosterone in OE-NPY^{DBH} and wildtype mice.[51] However, there were no differences between genotypes in baseline or stress-stimulated plasma corticosterone levels. Thus, overexpression of NPY in noradrenergic neurons does not seem to modulate the basal or stress-induced HPA-axis activity, and changes in corticosterone do not seem to contribute to the metabolic phenotype of OE-NPY^{DBH} mice.

Clinical Relevance

A link between NPY and human cardiometabolic diseases has been suggested in studies on a polymorphic form of *NPY* in humans.[69] The single amino acid polymorphism from leucine to proline (p.L7P, NM_000905.2:c.20 T >C; rs16139) has the highest prevalence (6-14%) in Nordic/Eastern European populations and is less common in Central or Southern European and rare in Asian and African American populations.[1,69,70] p.L7P is located in the exon 2 of the *NPY* gene. It is in the signal peptide, which is cleaved off from the mature protein. However, the polymorphism is functional or it is in linkage disequilibrium with another functional polymorphism.[71] *In vitro* and *in vivo* studies have shown that carriers of the p.L7P allele display altered cellular and plasma NPY concentrations.[72,73] The p.L7P seems to increase the processing and release of mature NPY from cells, whereas the wildtype L7L subjects produce more of the carboxy terminal containing form of the gene.[73,74] In line with this, P7 carriers showed normal plasma NPY levels at baseline, but increased secretion in response to sympathoadrenal stress of exercise.[73] These findings prompted the generation of the OE-NPY^{DBH} mice overexpressing NPY in noradrenergic neurons and showing increased plasma NPY levels in response to stress. Thus, the mouse was aimed to model the human polymorphism.

p.L7P has been associated with traits of the metabolic syndrome and vascular diseases associated with metabolic diseases in several study populations.[1] The associations of p.L7P with obesity are inconsistent with most studies showing no association.[1] However, there are interesting findings showing association of p.L7P with BMI in mostly non-obese study populations.[75,76] This suggests that elevated NPY increases adiposity before overt obesity, which is supported by the phenotype of OE-NPY^{DBH} mice: Increased WAT mass without increase in body weight. The mechanism for increased BMI in carriers of p.L7P is unclear as no differences in dietary intake have been detected.[76,77] OE-NPY^{DBH} mouse supports this finding by showing that NPY-induced increase in adiposity may occur without hyperphagia. Furthermore, the L7P polymorphism is associated with IGT and an earlier onset of type 2 diabetes in carriers of p.L7P,[78,79] especially in obese subjects.[80] Again the OE-NPY^{DBH} mice display with similar phenotype: IGT associated with obesity. Recently, it was reported that there is a gender difference in the NPY-mediated effects of the P7 allele.[81] The associations of the p.L7P seem to be more pronounced in men than women, which is in agreement with the findings in OE-NPY^{DBH} mice indicating that males develop more severe metabolic changes. The mouse also models the vascular diseases associated with the p.L7P, as it has been associated with hypertension[1,82] and shown to increase the risk of atherosclerotic vascular disease in middle-aged men,[83] patients with hypertension[84] and in type 1 and type 2 diabetic patients.[85,86]

Conclusions

To model human genetic variants and diseases with polygenetic origin in rodents is not straightforward. However, the OE-NPY^{DBH} mouse was able to mimic the metabolic and vascular profile associated with the polymorphic form of *NPY* in humans. The results show that NPY may induce metabolic diseases not only by stimulating appetite in the hypothalamus, but by promoting adiposity via brainstem and SNS pathways. Furthermore, the mouse model gives a novel tool to study the role of NPY in the development of the metabolic syndrome and vascular diseases. It can be exploited in basic research as well as in translational studies to develop and test potential therapies for human diseases.

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