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# Neuropeotide Y changes the excitability of fine afferent units in the rat knee joint

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**1** The aim of the present study was to examine the effects of the sympathetic co-transmitter Neuropeotide Y on primary afferent nerve fibres of the rat knee joint. The responses to passive joint rotations at defined torque were recorded from 41 slowly conducting afferent nerve fibres (0.9–18.8 m $s^{-1}$) innervating the knee joint capsule.

**2** About 70% of the joint afferents were significantly affected in their mechanosensitivity by topical application of Neuropeptide Y. Significant effects occurred at a concentration of 10 nM.

**3** Decreased mechanosensitivity was observed in about 40% of nerve fibres, whereas 30% of the units increased the mechanosensitivity. In addition, in about 35% of the fibres resting activity was induced or increased. Neither the conduction velocity nor the mechanical threshold of the units correlated with the described effects of Neuropeptide Y.

**4** NPY(13–36), a specific Y2-receptor agonist, only modulated the mechanosensitivity, with no effect on the resting activity. The effects on the mechanosensitivity were similar to Neuropeptide Y, i.e. increase and decrease of the response.

**5** Studies with the Y1-agonist ($Leu^{31}$, $Pro^{34}$)-NPY showed that activation of the Y1-receptor predominantly resulted in an enhanced mechanosensitivity and an induction or increase of a resting activity. The opposite effect was observed by application of BIBP 3226 BS, a Y1-receptor antagonist.

**6** In conclusion, these data indicate that Neuropeptide Y affects the excitability of sensory nerve fibre endings.

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**Keywords:** Neuropeptide Y; rat; nociception; mechanosensitivity; knee joint; sympathetic nervous system

**Abbreviations:** NPY, Neuropeptide Y

## Introduction

Neuropeotide Y (NPY) is widely distributed in the central and peripheral nervous system as well as in blood cells (Hökfelt *et al*., 1998; Ma & Bisby, 1998; Myers *et al*., 1988). This peptide is able to increase the excitability of neurones (Abdulla & Smith, 1999a,b) and is involved in the central modulation of feeding behaviour (Vezzani *et al*., 1999). NPY is also involved in the central modulation of nociceptive processes in rats and mice. However, excitatory as well as inhibitory effects of this peptide have been obtained (Broqua *et al*., 1996; Xu *et al*., 1999).

In the peripheral tissue, post-ganglionic sympathetic neurones co-release NPY with norepinephrine, intensifying the vasomotor response (Cortes *et al*., 1999; Lundberg *et al*., 1982). Besides this regulatory function, the post-ganglionic sympathetic system also interacts with primary afferents during pathophysiological processes such as nerve injury or tissue trauma (Jänig, 1996). While this effect is primarily based on interactions with α-adrenoreceptors, NPY may also affect sensory nerve fibres, as a proportion of dorsal root ganglion neurones exhibit mRNA for different NPY-receptor subtypes (Hökfelt *et al*., 1998).

A well-known *in vivo* model to study the effect of different mediators on the sensitivity of primary afferents in a peripheral tissue is the knee joint of the rat. Recently, it has been described that the mechanosensitivity of these afferents is modulated by different neuropeptides such as somatostatin, galanin and substance P (Heppelmann *et al*., 2000; Heppelmann & Pawlak, 1997a,b). The knee joint is also supplied with a great number of post-ganglionic sympathetic nerve fibres (Hildebrand *et al*., 1991), and it has been shown that NPY is present in bone and synovial fluid (Ahmed *et al*., 1994; Elfvin *et al*., 1998). In addition, during joint inflammation or rheumatoid arthritis the content of NPY markedly increased (Ahmed *et al*., 1994; Calza *et al*., 1998; Elfvin *et al*., 1998; Larsson *et al*., 1991).

Based on these data, it can be assumed that NPY released from sympathetic nerve fibres may affect the mechanosensitivity of primary afferent units. Therefore, the aim of the present study was to examine the effects of NPY on primary afferent nerve fibres in the rat knee joint. The importance of the different NPY-receptor subtypes was investigated by using specific agonists and antagonists for the Y1- and the Y2-subtype.

## Methods

### *General preparation*

The animals used in this study were acquired and treated in accordance with guidelines of the Council of the German

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Physiological Society, as well as regulations of the State of Bavaria, Germany; experiments were reviewed and consented to a local committee for animal care.

The study was carried out on 41 male Wistar rats (300–450 g), which were anaesthetized by an intraperitoneal injection of thiopentone (Trapanal, 100–120 mg $kg^{-1}$). The trachea was cannulated for artificial ventilation and to measure the actual $CO_2$-level. To maintain a deep anaesthesia of the animals throughout the experiment, additional doses of Trapanal (20–25 mg $kg^{-1}$) were injected through a catheter supplying the left femoral vein. The absence of any changes in blood pressure in response to noxious stimuli confirmed the depth of anaesthesia. The blood pressure was monitored with a pressure transducer *via* a catheter placed in the left femoral artery.

The right femur was fixed by a special grip. A pool was formed by skin flaps and filled with warm paraffin oil. In this pool the whole medial part of the right leg was exposed including the knee joint and the patellar ligament.

### *Experimental procedure*

Fine filaments of the proximal end of the saphenous nerve were dissected and put over a silver wire electrode, as described in earlier studies (Heppelmann & Pawlak, 1997a, b). Only afferent units with receptive fields on the knee joint capsule were investigated. The afferent terminal of the nerve fibre was electrically stimulated (5–15 V, 0.5 ms) within its receptive field to calculate the conduction velocity.

The mechanosensitivity of the nerve fibres were characterized during outward rotations of the knee joint using a torque-meter fastened at the hind paw of the rat. The knee joint was rotated with up to 60 mNm in steps of 10 mNm. Each step was held for 10 s before increasing the torque. This procedure was repeated every 3 min. (For details see Just *et al*., 2000).

### *Testing of substances*

At the beginning of the recording, a filter paper with a diameter of 5 mm was soaked with Tyrode solution and put onto the receptive field of the nerve fibre. The space between the paper and the tissue surface was additionally filled with a small drop of the solution. After at least five control movements, the paper was replaced by a new one soaked with different concentrations of NPY (1 nM–1 $\mu$M) in Tyrode solution. Each concentration was tested during six movement cycles.

In a second set of experiments this procedure was carried out with a specific $Y_1$-receptor agonist ((Leu$^{31}$, Pro$^{34}$)-NPY, Bachem) or a $Y_2$-receptor agonist (NPY (13–36); Bachem). The effects of these agonists were tested with concentrations between 10 nM and 1 $\mu$M. In most experiments both agonists were used, but in a different order.

Additional experiments were performed to test the possibility of a tonic release of NPY. Therefore the potent Y1-receptor antagonist BIBP 3226 BS (Boehringer Ingelheim, Germany) (Rudolf *et al*., 1994) was applied topically in a concentration of 10 $\mu$M, followed by the same concentration of (Leu$^{31}$, Pro$^{34}$)-NPY.

In the control movements the mechanical threshold of each fibre was determined. For a better comparison of the effects of the applied substances, the response of the units at 10 mNm above the threshold was used for statistical analysis. At least four responses from the control movements were averaged and set as 100%. Four to five responses after topical application of substances were averaged and compared to the control period using the Student's *t*-test. A *P*-value of less than 0.05 was set as criterion of significance. The data of all units were averaged and presented as mean ± s.e.mean.

At the end of the experiments, a filter paper with a small drop of 0.3 M KCl was placed onto the receptive field. In each case, the units responded with a burst of impulses indicating that the applied substances could reach the sensory endings. Thereafter the animals were sacrificed by injecting a lethal dose of Trapanal.

## Results

### *General observations*

The effects of NPY and specific NPY-receptor agonists were tested on 41 afferent nerve fibres which innervated the medial aspect of the joint. All units were classified as either C or A$\delta$ fibres, as their conduction velocities ranged from 0.9 to 18.8 m $s^{-1}$. The mechanical threshold tested in the way described ranged from about 10 to 50 mNm. All nerve fibres responded to passive outward rotations of the joint in a torque-dependent manner, i.e. increasing torque led to a greater response of the unit.

### *Effects of NPY on mechanosensitivity*

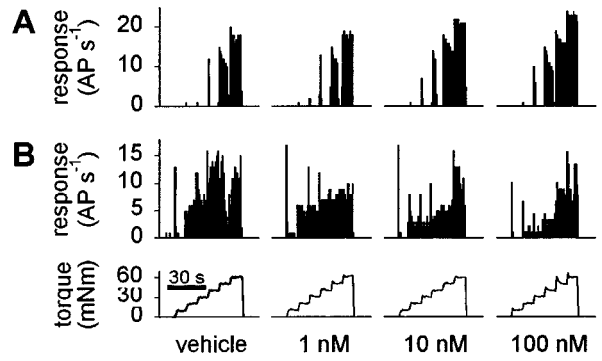
The effect of NPY was examined in 17 afferent nerve fibres. The excitability of some units was already affected at an application of 1 nM NPY, but the changes were not significant. A neuropeptide concentration of 10 nM caused marked changes in a proportion of the units. This modulatory effect of NPY was only augmented by a concentration of 1 $\mu$M.

Comparing the responses to knee joint rotation before and after the application of the peptide, only 29% (5/17) of the fibres did not change their mechanosensitivity. The remaining 71% (12/17) showed a significant change of the response to knee joint rotation. 29% (5/17) of the units increased their mechanosensitivity to about 160% of the control values (Figures 1, 4). Another 42% (7/17) decreased their response to about 53% of the control values (Figures 1, 4). This effect was most prominent at a torque close to the mechanical threshold (Figure 1A,B). Some of the fibres even changed their mechanical threshold.

Neither the conduction velocity ($P=0.28$; $r=-0.31$; Pearson product moment correlation) nor the mechanical threshold of the units ($P=0.52$; $r=-0.18$) correlated with the different effects of NPY (Figure 2).

### *Effect of NPY on resting activity*

In 35% (6/17) of the nerve fibres, NPY induced a resting activity of more than 0.1 Hz (3 units), or increased an already existing resting activity to values of up to 4.5 Hz (3 units) (Figures 3, 4). These effects were induced with a concentra-



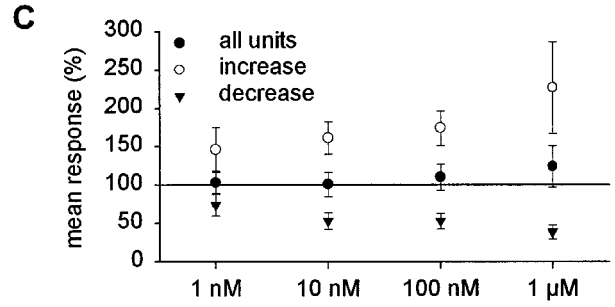




**Figure 1** Different effects of NPY on afferent nerve terminals. Examples of units are shown in which NPY lead to an increased (A) or decreased (B) mechanosensitivity in a dose dependent manner. Change of the responses at a torque of 10 mNm above the mechanical threshold are presented as the mean ± s.e.mean of the units with a significant increase and a significant decrease of the responses as percentage of the average control level that were set at 100% (C). The mean response of all units tested is presented as dots.

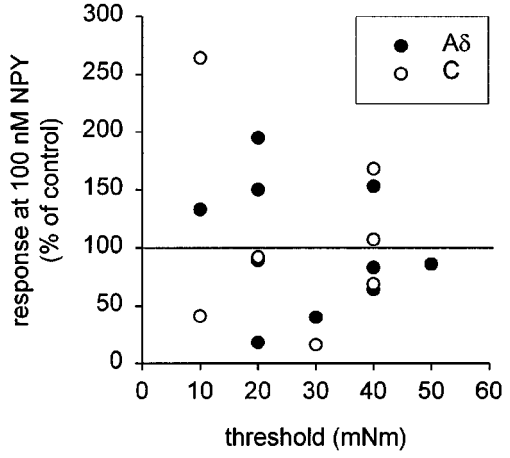



**Figure 2** Change of the mechanosensitivity after application of 100 nM NPY in dependence of the threshold of the unit. The respective NPY-effect was independent of the threshold and the classification of the afferent unit.

tion of 10 nM. Higher concentrations did not increase the resting activity further.

Resting activity and mechanosensitivity could be modulated independently, i.e. afferent nerve fibres which showed increased resting activity were not necessarily modulated in their mechanosensitivity and vice versa. Even fibres with

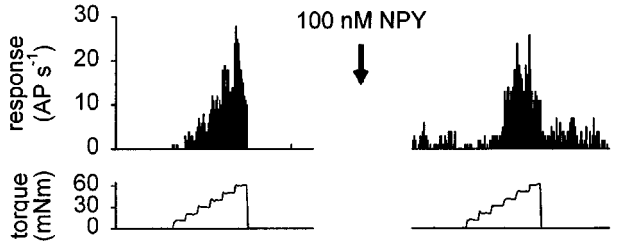



**Figure 3** Induction of a resting activity by a topical application of 100 nM NPY. The mechanosensitivity of this unit was slightly reduced at the same time.

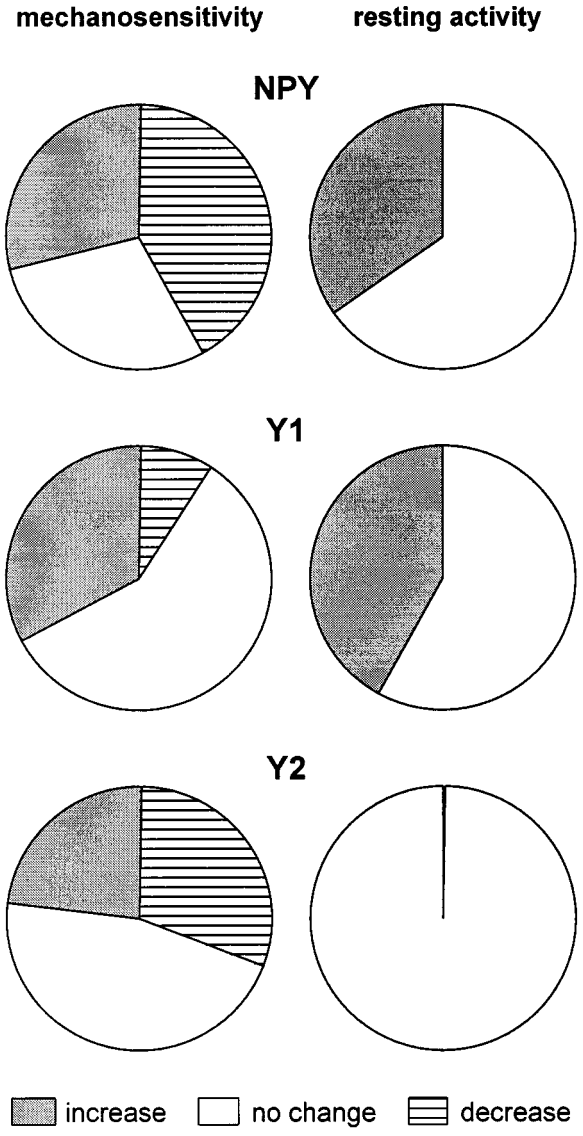



**Figure 4** Summary of the effect of NPY, the Y1- and the Y2-receptor agonist on the mechanosensitivity and resting activity of the examined nerve fibre ending. The effects of NPY and (Leu$^{31}$, Pro$^{34}$)-NPY (Y1) and NPY(13–36) (Y2) on the excitability of afferent knee joint units were tested. The percentage of units with no change (0, white), with increase (+, cross-hatched), and decrease (−, hatched) is represented.

increased resting activity and decreased mechanosensitivity after application of NPY were found.

### *Effects of specific receptor agonists and antagonists*

To examine whether the different effects of NPY on nerve fibre endings are based on distinct receptor subtypes, specific agonists to the Y1- and the Y2-receptor subtype were topically applied to additional 14 units with conduction velocities of 1.1–15.5 m $s^{-1}$. In most of the experiment both agonists were used but in different order (Y1: $n=12$; Y2: $n=13$). As all results obtained were independent of the order of application, the data of all examined units were combined.

After application of ($Leu^{31}$, $Pro^{34}$)-NPY, a specific agonist of the Y1-receptor, about 33% (4/12) of joint afferents significantly increased their mechanosensitivity, whereas only one fibre significantly decreased its response to outward rotation of the knee joint (Figure 4). The sensory nerve fibres were maximal affected at concentrations of at least 10 nM of the Y1-receptor agonist. At this concentration the mean response of all fibres to knee joint rotation approximately increased to 151±43% of the control value.

In about 42% (5/12) of the nerve fibres, resting activity was increased or induced by the Y1-receptor agonist to values of up to 3.8 Hz. Again, in these experiments resting activity and mechanosensitivity were independently affected.

Using NPY (13–36), a specific Y2-receptor agonist, the effects on joint units differed from that shown for activation of the Y1-receptor. Comparable to the results obtained with NPY maximal effects were found at concentrations of at least 10 nM. At this dose about 23% (3/13) of the units significantly increased their mechanosensitivity, whereas another 31% (4/13) of the sensory endings reduced their evoked responses (Figure 4). In contrast to the Y1-receptor agonist, NPY(13–36) had no influence on resting activity.

In an additional 10 experiments the effect of BIBP 3226 BS, a potent Y1-receptor antagonist was tested. In 3 units the mechanosensitivity was decreased significantly. The effect could at least partially be antagonised by topical application of ($Leu^{31}$, $Pro^{34}$)-NPY (Figure 5).

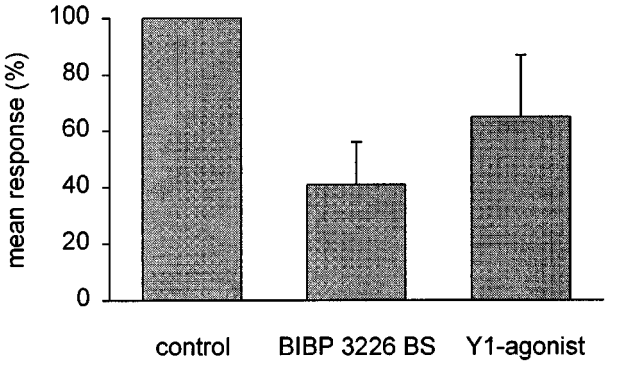



**Figure 5** Effect of BIBP 3226 BS on the mechanosensitivity. In 3 of 10 units the mechanosensitivity was significantly reduced after topical application of BIBP 3226 BS. The effect was antagonized by topical application of ($Leu^{31}$, $Pro^{34}$)-NPY. Changes of the responses at a torque of 10 mNm above the mechanical threshold is presented as the mean±s.e.mean. The response to control movements was defined as 100%.

## Discussion

The present results show two effects of NPY on slowly conducting sensory units of the rat knee joint: (1) increase or decrease of the mechanosensitivity, and (2) induction or increase of a resting activity.

### *NPY and mechanosensitivity*

Studies on the processing of nociceptive information in the central nervous system have shown that NPY may be involved in nociception. However, this peptide revealed inhibitory as well as excitatory actions, but the antinociceptive effects were more focused. Intracerebroventricular or intrathecal administration of NPY or analogues of this peptide showed antinociceptive effects in the mouse writhing (Broqua *et al*., 1996) or the hot plate test (Hua *et al*., 1991). In flexor reflexes intrathecal application of NPY causes dose dependently a biphasic effect with inhibition at low doses and facilitation at higher doses. This biphasic effect was shown in normal rats as well as after an inflammation or an axotomy (Xu *et al*., 1998; 1999). Exclusively excitatory effects of NPY and its analogues were described after nerve injury (Abdulla & Smith, 1999; White, 1997).

In the present study, there were both effects as well. Marked increases in the mechanosensitivity were found in a proportion of joint afferents, whereas another group showed significant decreases. This may be explained by different receptor subtypes present at the sensory endings. However, an application of the Y2-receptor agonist also revealed both kinds of change. Only the Y1-receptor agonist predominantly showed significant increases of the mechanosensitivity in a proportion of the examined units.

Another explanation for the different effects could be that NPY also affects the sensory endings indirectly *via* activation of other cells. This may be the case for the effect of the Y1-receptor agonist. Although about 33% of all small dorsal root ganglion cells contain the Y1-receptor, only a small amount of this receptor subtype is transported into the peripheral nerve fibres (Zhang *et al*., 1994).

It is very unlikely that changes of mechanosensitivity can be attributed to NPY-dependent vasoconstriction, as Shakhanbeh & Lynn (1993) showed that changed skin blood flow did not influence the mechanosensitivity of slow conducting afferent nerve fibres. In addition, NPY always decreases the local blood flow, while the mechanosensitivity of afferent units of the rat knee joint can increase or decrease after topical application of NPY. It is doubtful that the contrary effects on mechanosensitivity can solely be explained by vasomotor responses of only one direction. However, a partial influence of vasoconstriction on the mechanical excitability of afferent units of the knee joint can not fully be excluded.

The activation of the Y1-receptor leads to an increased mechanosensitivity, while the excitability is reduced after blocking of this receptor. The latter may indicate a tonic release of NPY into the knee joint. This tonic release can not be caused by an activation of the sympathetic nerve fibres, as the saphenus nerve has been cut proximal to the recording site. Consequently the tonic release of NPY must be independent of action potentials of the sympathetic nerve

fibres. But there may be further sources of NPY as it was shown that for example also platelets can be activated to release this neuropeptide (Myers *et al*., 1988).

If one combines the responses of all examined units, there was no significant change in comparison with the control values, although a concentration of 1 $\mu$M NPY leads to a slight increase of the mechanosensitivity (Figure 1C, dots). Based on the hypothesis that the whole sensory input from the knee joint is necessary for its processing within the central nervous system, these data would mean that there is no significant influence of NPY. However, the phenomenon that only a proportion of primary afferents is affected by different substances is found in many studies considering the action of inflammatory mediators (Heppelmann & Pawlak, 1997a,b; Schaible & Grubb, 1993). In addition, an application of galanin reduced the mechanosensitivity in about 40% of knee joint afferents, whereas in about 10% there was a significant increase (Heppelmann *et al*., 2000). Based on these data, it may also be speculated that aside from the activity pattern of primary afferent neurones, the spinal hook up also plays an important role for the central interpretation of the afferent input.

### *NPY and resting activity*

Another important finding of this study was the induction or increase of a resting activity in a proportion of joint afferents. NPY never had an inhibitory effect on existing resting activity. It has already been shown that an activation of the sympathetic nervous system leads to discharge in myelinated and in unmyelinated nociceptive sensory nerve fibres (Hu & Zhu, 1989; Roberts & Elardo, 1985a,b; Roberts *et al*., 1985). The effects have mainly been attributed to interactions of norepinephrine with $\alpha$-receptors. Influences of NPY could not be excluded, as this peptide is co-localized with norepinephrine within peripheral neurones (Lundberg *et al*., 1982). In addition, pathophysiological pain states in diseases are accompanied with increased NPY concentration. For example the NPY concentration in synovial fluid increases in patients with painful rheumatoid arthritis (Larsson *et al*., 1991). An increased NPY content also occurs in dorsal root ganglion and dorsal horn neurones after inflammation or nerve injury (Ma & Bisby, 1998). Therefore, NPY may also be involved in the activation of the sensory nerve fibre endings by a binding on the Y1-receptor. Similar to the effects of NPY on the mechanosensitivity, it can not be excluded, that also the resting activity is increased indirectly *via* activation of other cell types.

The effect on resting activity was independent of effects on the mechanosensitivity. This finding may indicate that there are two separate mechanisms. Mechanosensitivity may depend on mechanosensitive membrane channels that are regulated by a variety of mediators including NPY. Resting activity, however, may be based on the regulation of other ion channels which influence the membrane potential. Altered properties of different membrane channels may depolarize the sensory endings and induce action potentials.

Just like mechanosensitivity, resting activity also seems to be independent of vasoconstriction forced by NPY, as the background firing of nociceptors remains constant even after total arterial ligation (Lynn, 1979).

In conclusion, the present data show that the sympathetic nervous system and other sources of NPY seem to influence the mechanosensitivity of articular afferent units directly or indirectly. As the Y1-receptor agonist predominantly increased the mechanosensitivity and induced or increased a resting activity, this receptor subtype may be a target for the development of new analgesic agents.

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