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Involvement of neuropeptide Y and Y1 receptor in antinociception in the arcuate nucleus of hypothalamus, an immunohistochemical and pharmacological study in intact rats and rats with inflammation

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Abstract

Neuropeptide Y (NPY) plays an important role in pain modulation at different levels in the central nervous system. In the brain, NPY and NPY receptors distribute abundantly in the arcuate nucleus of hypothalamus (ARC), a structure involved in pain processing. The present study was undertaken to investigate the role of NPY in nociceptive modulation in the ARC of intact rats and rats with carrageenan-induced inflammation. Intra-ARC administration of NPY induced dose-dependent increases in hindpaw withdrawal latencies (HWLs) to thermal and mechanical stimulation in intact rats, which was attenuated by the Y1 receptor antagonist NPY28-36. Intra-ARC administration of NPY also induced dose-dependent increases in HWLs to noxious stimulation in rats with inflammation. Furthermore, intra-ARC injection of either the antiserum against NPY or NPY28-36 induced decreases in HWLs in rats with inflammation, while both of them produced no effects in intact ones. Additionally, there were marked increases of Y1 receptor in the bilateral ARC of rats with inflammation tested by immunohistochemistry, while no significant changes of NPY were observed, implicating that the increased Y1 receptor has an important effect in the NPY-induced antinociception. We also found that intra-ARC injection of Y2 receptor agonist NPY3-36 produced no significant antinociception in either intact rats or rats with inflammation. Together, we demonstrate that NPY exerts an antinociceptive effect in the ARC of intact rats and rats with inflammation. Both Y1 receptor and endogenous released NPY in the ARC are involved in the nociceptive modulation during inflammation.

Keywords: Neuropeptide Y; Y1 receptor; Hindpaw withdrawal latency; Arcuate nucleus; Nociceptive modulation; Inflammation

1. Introduction

Inflammation is characterized by spontaneous pain, hyperalgesia and allodynia (Porreca et al., 2002; Woolf and Costigan, 1999; Woolf and Salter, 2000). Plasticity of various molecules, neuropeptides and neurotransmitters exerts an important effect in the nociceptive modulation during inflammation (Buritova et al., 1998; Dubner and Ruda, 1992). One of those adapted molecules is neuropeptide Y (NPY), a 36-amino-acid neuropeptide, that mediates its diverse biological functions through activation of six different G-protein-coupled receptors. The effect of NPY on nociceptive modulation has been extensively studied and Y1 receptor has been proved to be required for the central physiological and pharmacological NPY-induced analgesia (Li et al., 2002; Silva et al., 2002).

The role of NPY in nociceptive modulation during inflammation has been implicated by the neurochemical plasticity and the endogenous antinociceptive response of NPY to inflammatory pain at the spinal level (Ji et al., 1994; Taiwo and Taylor, 2002). Ji and colleagues found that there are increases of NPY and Y1 receptor in the dorsal horn of rats with complete Freund's adjuvant (CFA)-induced inflammation (Ji et al., 1994). The up-regulated expression of NPY system has been suggested to be associated with increased NPY release, inhibition of nociceptive

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transmission, and ultimately a reduction in the severity of inflammatory hypersensitivity (Ji et al., 1994). Furthermore, Taiwo and Taylor (2002) showed that endogenously released NPY exerts a tonic antinociceptive effect at the spinal level of rats with inflammation, which is mediated by Y1 receptor. Moreover, a recent study found that Y1 receptor knockout mice displays no sign of neurogenic inflammation, which indicates a critical role of endogenous NPY during inflammation and leads us to question the possible role of NPY in the brain (Naveilhan et al., 2001).

Strong evidence supports the idea that manifestations of inflammation require activating supraspinal structures (Dubner and Ren, 1999; Porreca et al., 2002; Ren and Dubner, 1996; Urban and Gebhart, 1999). In the brain, the arcuate nucleus of hypothalamus (ARC) particularly interests us. There are large projections from the ARC to two major relay stations: the periaqueductal gray (PAG) and the nucleus raphe magnus involved in descending inhibitory pathways (Millan, 2002; Sim and Joseph, 1991; Stamford, 1995). The ARC has an important role in pain regulation, especially in inflammatory pain (Bach, 1997; Sun et al., 2003; Zangen et al., 1998). Furthermore, there are dense distributions of NPY and NPY receptors in the ARC (Migita et al., 2001; Silva et al., 2002). Thus, the present study was carried out to elucidate the role of NPY and its receptors in nociceptive modulatiuon in the ARC of intact rats and rats with inflammation.

2. Materials and methods

2.1. Animal preparation

Experiments were carried out on freely moving male Wistar rats weighting between 220–250 g (Experimental Animal Center of Peking University, Beijing, China). The rats were housed in cages with free access to food and water, and maintained in a room temperature of 24 ± 2 °C with a normal day/night cycle. All experiments were conducted according to the guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and every effort was made to minimize animal suffering and the number of animal used.

2.2. Carrageenan-induced inflammation

Carrageenan-induced inflammation is a widely used method that can closely reproduce some human pain syndromes (Di Rosa and Sorrentino, 1968). Animals received a unilateral injection of carrageenan (2 mg in 0.1 ml saline; Sigma, St Louis, MO) into the left hindpaw, and the contralateral paw was untreated. Three hours after injection of carrageenan, the hindpaw withdrawal latency (HWL) was measured by the hot plate Test and the Randall Selitto Test. Then each animal received an intra-ARC injection of either vehicle or drug, and the HWLs of each animal were thereafter assessed at 5, 10, 15, 20, 30 and 60 min.

2.3. Intra-ARC injection

The animals were anaesthetized by intraperitoneal (i.p.) sodium pentobarbital (50 mg/kg) and were mounted on a stereotaxic instrument. A stainless steel guide cannula (20 gauge) was positioned 1.8 mm dorsally to the ARC (B -3.8, L 0.4-0.5, V 9.8 mm; B, Bregma; L, lateral to midline; V, ventral to the surface of skull) according to Paxinos and Watson (1998) and was fixed to the skull by dental acrylic. On the experiment day, a stainless steel needle (26 gauge) was inserted directly into the guide cannula, with 1.8 mm beyond the cannula tip. One microliter of solution was thereafter infused into the ARC over 1 min, and the injection needle was left in the place for 30 s after each injection. Solutions for intra-ARC injection were prepared with sterilized saline (0.9%), each with a volume of 1 μl of: (1) 0.01, 0.02, 0.05, 0.1 or 0.5 nmol of NPY (human NPY, Neosystem, France); (2) 0.1, 0.5 or 1 nmol of NPY28-36 ((Pro30, Try32, Leu34) NPY28-36, Neosystem, France); (3) 0.5 or 1.0 nmol of NPY3-36 (Neuropeptide Y (3-36), Neosystem, France); (4) 0.01, 0.05 or 0.1 μl of rabbit antiserum against NPY (Phoenix pharmaceuticals, Belmont, CA), 0.1 μl of normal rabbit serum as a control.

NPY28-36 has been widely used as Y1 receptor antagonist in many studies (Berglund et al., 1999; Sharma et al., 1999). It binds with high affinity to Y1 receptor and with poor affinity to Y2 receptor in rat brain (Leban et al., 1995). NPY3-36 is a selective Y2 receptor agonist and has a 1000 fold lower affinity than NPY for Y1 receptor on SK-N-MC cells (Grandt et al., 1996).

2.4. Nociceptive tests

Rats were accustomed to the testing conditions for 5 days before the starting of the experiment to minimize the stress induced by handling. The HWLs during thermal and mechanical stimulation were measured as described previously (Yu et al., 1996; Zhou et al., 2003). Briefly, the entire ventral surface of the rat hindpaw was placed manually on a hot plate, which was maintained at a temperature of 52 °C (51–53 °C). The time to hindpaw withdrawal was measured in seconds and referred to as the HWL to thermal stimulation. The Randall Selitto Test (Ugo Basile, Type 7200, Italy) was used to assess the HWL to mechanical stimulation. A wedge-shaped pusher at a loading rate of 30 g/s was applied to the dorsal surface of the manually handled hindpaw and the latency required to initiate the withdrawal response was assessed and expressed in seconds. The average value of the HWL obtained before intra-ARC injection was regarded as the basal HWL. Then intra-ARC injection was performed over 1 min. The HWLs recorded during subsequent experiments were expressed as percentage changes of the basal level for each rat (% change of the HWL). Each rat was tested with both types of stimulation. Every measurement of the HWL to both thermal and mechanical stimulation was finished within 2 min and the intervals were enough for rats to recover from stress. A cut-off limit of 15 s was set up to avoid tissue damage.

2.5. Inclined plane test

The inclined plane test was performed according to Rivlin and Tator (1977). Briefly, the rat was placed on an inclined plane, which can be adjusted to provide a slope of varying grade, and the maximum angle of the plane at which the rat can maintain its

position without falling in 5 sec was recorded to assess the limb muscle strength of the rat. The maximum angles tested before the injection is regard as the basal angle (100%), and the angle tested after intra-ARC injection were expressed as percentage of the basal level for each rat (% of the basal angle).

2.6. Immunohistochemistry and images analysis

Animals were studied 4 h after the injection of carrageenan ($n=4$), at a time-point where marked signs of hyperalgesia were present. Intact rats were used as a control ($n=4$). The animals were anesthetized with sodium pentobarbital at a lethal dose (90 mg/kg, i.p.) and euthanized by transcardiac perfusion (saline wash, followed by 4% paraformaldehyde in 0.1 M phosphate-buffer, pH 7.4). The rat brains were removed and postfixed in the above solution for 3 h, and then stored in 0.1 M phosphate-buffered saline (PBS) containing 30% sucrose for at least 24 h for cryoprotection. Frozen sections were cut with a freezing microtome at 30 μm and collected for floating immunostaining.

Immunohistochemistry was performed with an avidin-biotin complex (ABC) Elite Kit (Vector, Burlingame, CA), as described by Sun et al. (2004). All sections for each specific staining were performed under the same conditions. First, the sections were preincubated for 30 min in PBS containing 0.3% Triton X-100, supplemented with 10% normal goat serum at room temperature. Then, the sections were processed for immunohistochemistry with rabbit antiserum against NPY or rabbit antiserum against Y1 receptors (#96106; provided by Dr. Ohning, CURE/UCLA, Los Angeles, CA), diluted 1:1000. After incubation for 36 h with primary antibody (4 °C) and for 1 h in goat anti-rabbit biotinylated secondary antibody (at room temperature; Vector; 1:200), the reaction was developed with the avidin-biotin peroxidase protocol (ABC Kit; Vector), with 3, 3′-diaminobenzidine as chromogen. Control incubations with primary antiserum omitted were included and resulted in a lack of specific staining (data not shown).

The rabbit antiserum against Y1 receptor C-terminal portion was used in the present study. The specificity of this antiserum has been illustrated in a previous study (Kopp et al., 2002). It has been demonstrated that after the preabsorption of Y1 receptor antiserum with the immunogenic Y1 peptide, none of the Y1 receptor immunoreactive patterns were observed in the immunochemical study in rat brain. Also, the Y1 receptor positive structures could not be detected in the immunostaining study in Y1 receptor knockout mouse brain (Kopp et al., 2002).

The density of NPY and Y1 receptor immunoreactivity in the ARC in both intact rats and rats with inflammation was determined with NIH-image program Image J (http://rsb.info.nih.gov/ij/. Mizushima et al., 2005; Multon et al., 2005; Novakovic et al., 1999; Pezet et al., 2001). Digitized images of sections in the ARC were captured with a high resolution CCD camera (JVC TK-C1380/TK-C1381). Each image was digitized with 256 gray levels with a value of 0 for a white pixel and 255 for a black pixel (Ng and Ong, 2001; Novakovic et al., 1999). To quantify the density of staining, we established a threshold above which mean gray value of the area was calculated (Chacur et al., 2004; Ma et al., 2005; Malmberg and Basbaum, 1998; Multon et al., 2005; Pezet et al., 2001). The number of pixels and the average gray values above the set background were then computed and multiplied, giving an integrated densitometric measurement. Separate measurements were made for each ARC section (Chacur et al., 2004; Pezet et al., 2001). Every fifth section of a series of ARC sections from each rat was evaluated. Four rats (6 sections from each rat) were analyzed (Zhou et al., 2003). An observer blinded to the treatment carried out the density measurements.

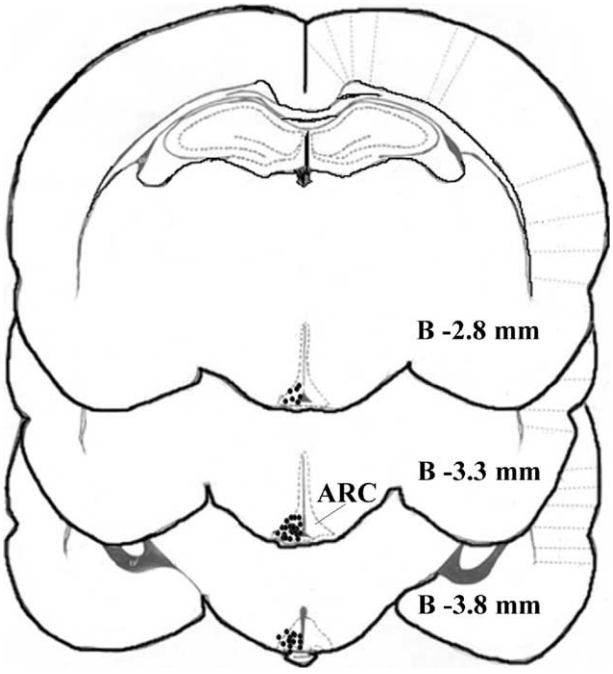

Fig. 1. Illustration of the location of the tip of the injection needle in the ARC.

2.7. Statistical analysis

At the end of the experiments, the location of the tip of the injection needle was verified (Fig. 1). Only the results from nociceptive tests where the tips of the injection needle were within the ARC were used for statistical analysis in behavioral experiments.

All the data were presented as mean ± SEM. Statistical differences between groups were determined by two-way analysis of variance (ANOVA) for repeated measurements in behavioral experiments and by Student's two-tailed *t*-test in immunohistochemical results and inclined plane test. The ED50 values were determined by regression analysis. The shift factors were determined by ED50 dose ratios as described before (Gray et al., 1999). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ were considered as significant differences.

3. Results

3.1. Antinociceptive effects of intra-ARC administration of NPY in intact rats

Based on the abundant distribution of NPY and NPY receptors in the ARC, it is quiet possible that NPY is involved in pain modulation in the ARC. In order to test whether NPY has a role in nociceptive modulation in the ARC of intact rats, four groups of intact rats received

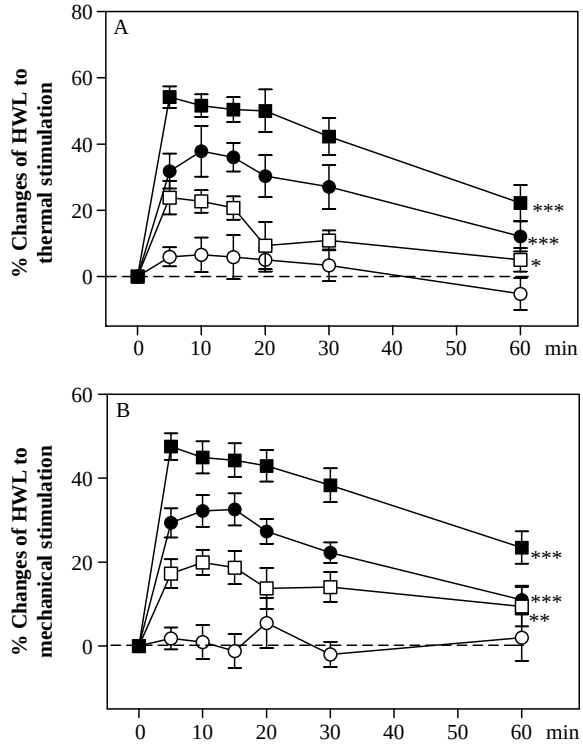

Fig. 2. Effects of intra-ARC injection of NPY on HWLs to thermal (A) and mechanical stimulation (B) in intact rats. 1 μl of 0.9% saline (○); 0.02 nmol (□); 0.1 nmol (●); 0.5 nmol of NPY (■). The statistical difference between groups was determined by two-way ANOVA (*$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared with the control group).

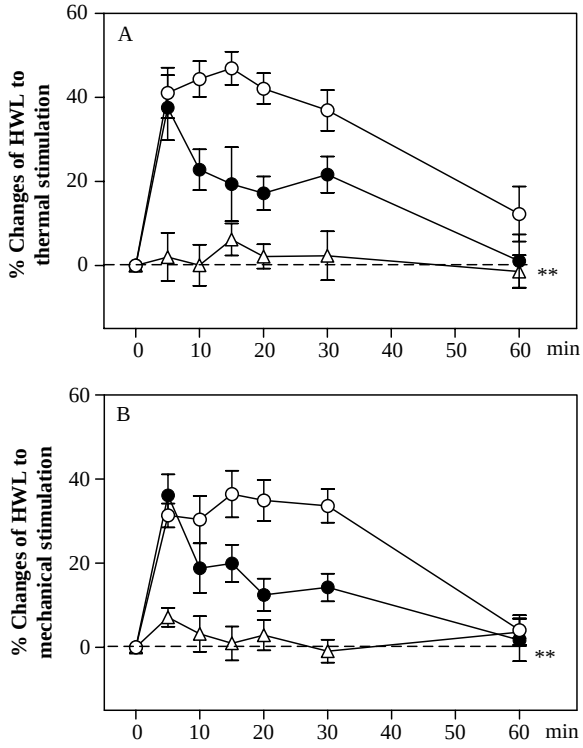

Fig. 3. Blockade of intra-ARC injection of NPY28-36 on the NPY-induced increases in HWLs to thermal (A) and mechanical stimulation (B) in intact rats. Time = 0 min: intra-ARC injection of 0.5 nmol of NPY, or NPY3-36; time = 5 min: intra-ARC injection 0.5 nmol of NPY28-36, or 0.9% saline as the control group. NPY + 0.9% saline 1 μl (○); NPY + 0.5 nmol of NPY28-36 (●); Saline + 0.5 nmol of NPY28-36 (△). Only the data measured at 10, 15, 20, 30 and 60 min were taken for two-way ANOVA (**$P<0.01$ compared with the control group).

intra-ARC injection of 0.02 ($n=7$), 0.1 ($n=7$) or 0.5 ($n=8$) nmol of NPY, and 1 μl of 0.9% saline as a control ($n=7$).

As shown in Fig. 2, the HWLs to thermal and mechanical stimulation increased significantly after intra-ARC injection of 0.02 nmol (Thermal test: $F_{(1,12)}=7.94$, $P<0.05$; Randall Selitto test: $F_{(1,12)}=16.94$, $P<0.01$), 0.1 nmol (Thermal test: $F_{(1,12)}=18.83$, $P<0.001$; Randall Selitto test: $F_{(1,12)}=52.95$, $P<0.001$) or 0.5 nmol of NPY (Thermal test: $F_{(1,13)}=68.28$, $P<0.001$; Randall Selitto test: $F_{(1,13)}=128.04$, $P<0.001$). The results demonstrate that intra-ARC administration of NPY induced dose-dependent increases in HWLs to thermal and mechanical stimulation, indicating that NPY exerts an antinociceptive effect in the ARC of intact rats.

3.2. Involvement of Y1 receptor in the NPY-induced antinociception in the ARC

To determine whether the effect of NPY was mediated by Y1 receptor, the Y1 receptor antagonist NPY28-36 was used. One group of intact rats received intra-ARC injection of 0.5 nmol of NPY, followed 5 min later by intra-ARC injection of 0.5 nmol of NPY28-36 ($n=7$) or 1 μl of 0.9% saline ($n=7$) as a control (Fig. 3). The HWLs to both thermal and mechanical stimulation increased after intra-ARC injection of NPY. Furthermore, the increased HWLs were significantly attenuated by subsequent intra-ARC injection of NPY28-36 (Thermal test: $F_{(1,12)}=12.04$, $P<0.01$. Randall Selitto test: $F_{(1,12)}=9.51$, $P<0.01$). Another group of intact rats ($n=6$) received intra-ARC injection of 1 μl of saline, followed 5 min later by intra-ARC injection of 0.5 nmol of NPY28-36. There were no significant changes in HWLs during 60 min after the injection, indicating that there is no tonic effect of NPY in the ARC of intact rats.

NPY28-36 is also a weaker antagonist of Y2 receptor (Leban et al., 1995). Thus, to test whether Y2 receptor is responsible for the NPY-induced analgesia in the ARC, there were another group of rats ($n=5$) received intra-ARC injection of 0.5 nmol of NPY3-36. Fifteen minutes after intra-ARC administration of NPY3-36, there were no significant alterations in HWLs to both the thermal ($0.13\pm0.96\%$) and mechanical ($4.34\pm3.10\%$) stimulation. The same dose of NPY produced an antinociceptive effect of $50.44\pm3.73\%$ and $44.30\pm4.03\%$ in hot plate test and Randall Selitto test respectively (obtained 15 min after the microinjection). This suggests that Y2 receptor does not participate in the antinociceptive effect of NPY in the ARC of intact rats. Together, the present results indicate that Y1 receptor, not Y2 receptor, is involved in the NPY-induced antinociception in the ARC of intact rats.

3.3. Antinociceptive effects of intra-ARC administration of NPY in rats with inflammation

We further test whether NPY also participates in the nociceptive modulation during inflammation. Four groups of rats with carrageenan-induced inflammation received intra-ARC injection of 0.01 ($n=6$), 0.05 ($n=6$) or 0.5 ($n=6$) nmol of NPY, or 1 μl of 0.9% saline ($n=6$) as a control (Fig. 4).

The HWLs to both thermal and mechanical stimulation increased significantly after intra-ARC injection of 0.05 nmol (Thermal test: $F_{(1,10)}=3.99$, $P=0.07$. Randall Selitto test: $F_{(1,10)}=8.15$, $P<0.05$), 0.5 nmol (Thermal test: $F_{(1,10)}=50.67$, $P<0.001$. Randall Selitto test: $F_{(1,10)}=71.69$, $P<0.001$) of NPY, but not 0.01 nmol of NPY (Thermal test: $F_{(1,10)}=1.04$, $P=0.33$. Randall Selitto test: $F_{(1,10)}=0.16$, $P=0.70$). The results prove an antinociceptive effect of NPY in the ARC of rats with inflammation.

3.4. Comparison of the antinociceptive effects of NPY in the ARC of intact rats and rats with inflammation

In order to compare the antinociceptive effect of NPY in intact rats and rats with inflammation, the Dose–response (D-R) curve was conducted (Fig. 5). At lower doses, the doses of NPY used in intact rats (0.02 and 0.1 nmol) and rats with inflammation (0.01 and 0.05 nmol) were not same, thus, it was difficult to compare the increased HWLs between the two groups. At high dose (0.5 nmol), NPY produced a pronounced antinociception to mechanical stimulation in the ARC of rats with inflammation ($t=12.52$, $P<0.01$), and no significant changes to thermal stimulation were observed ($t=0.43$, $P=0.51$).

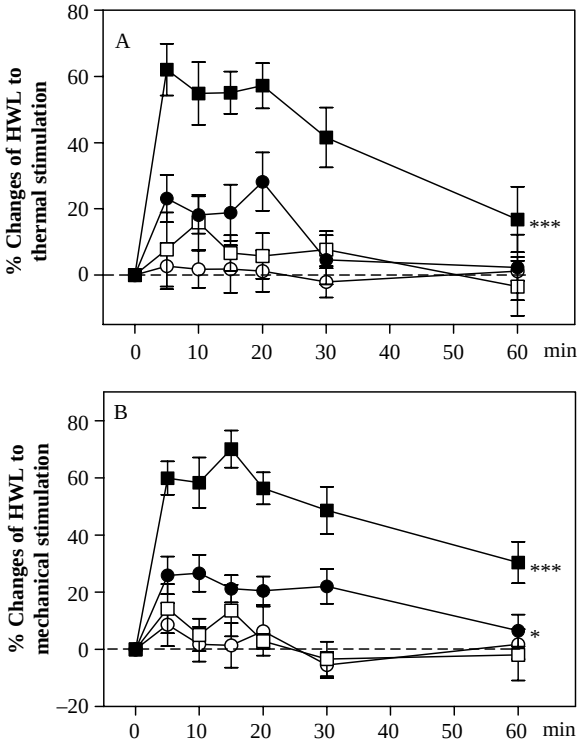

Fig. 4. Effects of intra-ARC injection of NPY on HWLs to thermal (A) and mechanical stimulation (B) in rats with inflammation. 1 μl of 0.9% saline (○); 0.01 nmol (□); 0.05 nmol (●); 0.5 nmol of NPY (■). The statistical difference between groups was determined by two-way ANOVA (*$P<0.05$ and ***$P<0.001$ compared with the control group).

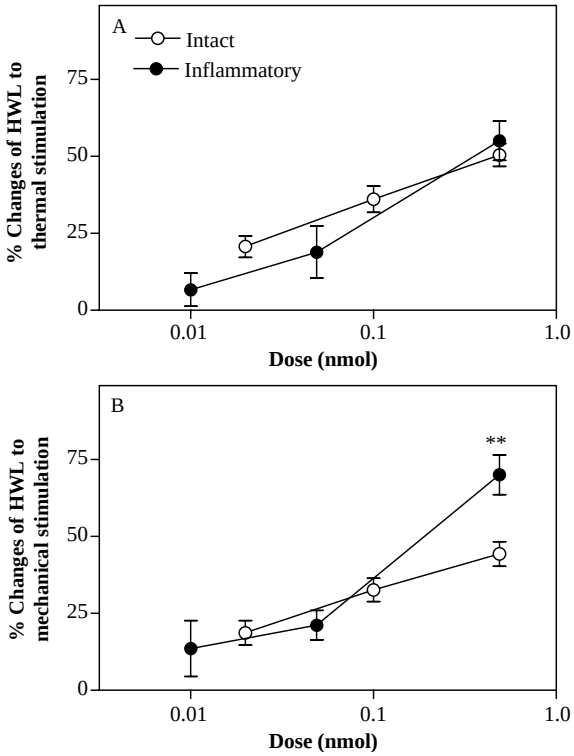

Fig. 5. Dose–response effects for NPY in intact rats (ED50 in thermal test: 0.39 nmol, 0.27–0.57; ED50 in mechanical test: 0.83 nmol, 0.53–1.31) and rats with inflammation (ED50 in thermal test: 0.38 nmol, 0.34–0.41; ED50 in mechanical test: 0.19 nmol, 0.06–0.59). The nociceptive responses of rats were assessed at 15 min after the injection. There were 6–8 rats per group. The statistical difference between groups was determined by Student two-tailed t-test (**$P<0.001$ compared with the control group).

The ED50 values were also calculated to determine the dose relationship of NPY between intact rats and rats with inflammation. In the hot plate test, the ED50s for NPY analgesia were similar in intact rats (0.39 nmol, 95% confidence interval: 0.27–0.57 nmol) and rats with inflammation (0.38 nmol, 95% confidence interval: 0.34–0.41 nmol). However, in the Randall Selitto Test, the ED50s of the D-R curve of inflammatory rats (0.19 nmol, 95% confidence interval: 0.06–0.59 nmol) were less than that observed in intact ones (0.83 nmol, 95% confidence interval: 0.53–1.31 nmol), shifting to the left by a factor of 0.23.

3.5. Influence of intra-ARC administration of the antiserum against NPY on the inflammation-induced hyperalgesia

It has been demonstrated that endogenous NPY modulates nociceptive transmission at spinal levels of inflammatory animals (Taiwo and Taylor, 2002). To test

the possible nociceptive role of endogenous NPY in the ARC during inflammation, four groups of rats with inflammation received intra-ARC injection of 0.01 ($n=6$), 0.05 ($n=7$) or 0.1 ($n=7$) μl of the antiserum against NPY, or 0.1 μl of normal rabbit serum as a control ($n=6$). Results are shown in Fig. 6.

The HWLs to noxious stimulation decreased significantly after intra-ARC injection of 0.01 μl (Thermal test: $F_{(1,10)}=18.06$, $P<0.01$. Randall Selitto test: $F_{(1,10)}=6.10$, $P<0.05$), 0.05 μl (Thermal test: $F_{(1,11)}=29.60$, $P<0.001$. Randall Selitto test: $F_{(1,11)}=3.71$, $P=0.08$) or 0.1 μl of the antiserum against NPY (Thermal test: $F_{(1,11)}=49.10$, $P<0.001$. Randall Selitto test: $F_{(1,11)}=86.80$, $P<0.001$).

Intact rats ($n=6$) also received intra-ARC injection of 0.1 μl of the antiserum against NPY. There were no significant changes in HWLs to noxious stimulation (data not shown). The results indicate an antinociceptive role of tonic released endogenous NPY in the ARC of rats with inflammation.

3.6. Influence of intra-ARC administration of NPY28-36 or NPY3-36 on the inflammation-induced hyperalgesia

Four groups of rats with inflammation received intra-ARC injection of 0.1 ($n=6$), 0.5 ($n=6$) or 1 ($n=6$) nmol of NPY28-36, and 1 μl of 0.9% saline as a control ($n=6$). As shown in Fig. 7, the HWLs to thermal and mechanical stimulation decreased significantly after intra-ARC injection of 0.1 nmol (Thermal test: $F_{(1,10)}=13.79$, $P<0.01$. Randall Selitto test: $F_{(1,10)}=6.24$, $P<0.05$), 0.5 nmol (Thermal test: $F_{(1,10)}=21.70$, $P<0.001$. Randall Selitto test: $F_{(1,10)}=15.00$, $P<0.01$) or 1 nmol of NPY28-36 (Thermal test: $F_{(1,10)}=42.70$, $P<0.001$. Randall Selitto test: $F_{(1,10)}=16.20$, $P<0.01$).

Another group of rats with inflammation ($n=5$) received intra-ARC administration of 1 nmol of NPY3-36. We found that NPY3-36 produced no significant antinociception (Thermal test: $2.61\pm5.23\%$ changes of HWLs. Randall Selitto test: $0.72\pm3.02\%$ changes of HWLs) at 15 min after the microinjection. The results suggest that the antinociceptive effect of endogenous NPY is mediated by Y1 receptor.

3.7. Influence of intra-ARC injection of NPY or NPY28-36 on the inclined planet test

In order to eliminate the possibility of reduced motor activity due to intra-ARC injection of NPY and NPY28-36, motor performance of the rats was evaluated by inclined plane test (Follett et al., 2004; Rivlin and Tator, 1977). We found that intra-ARC administration of 0.5 nmol of NPY or 0.5 nmol of NPY28-36 did not alter the motor performance

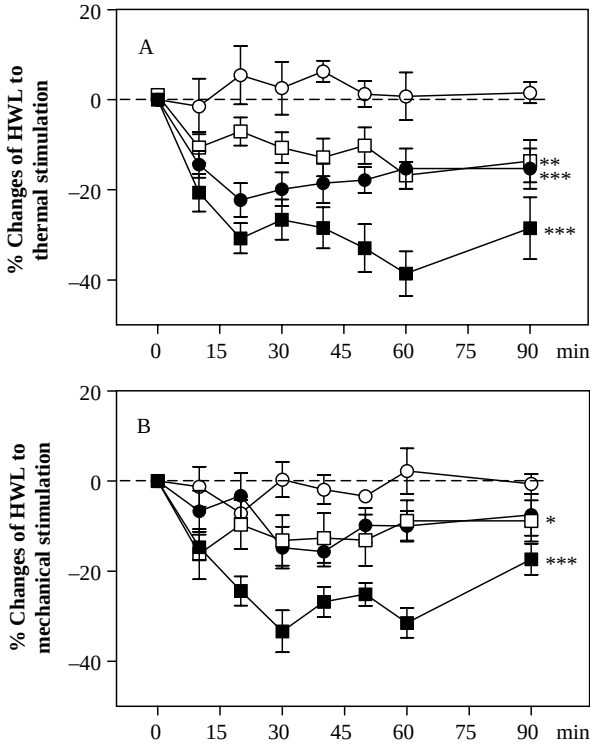

Fig. 6. Effects of intra-ARC injection of the antiserum against NPY on HWLs to thermal (A) and mechanical stimulation (B) in rats with inflammation. 0.1 μl of normal serum (○); 0.01 μl (□); 0.05 μl (●); 0.1 μl of antiserum against NPY (■). The statistical difference between groups was determined by two-way ANOVA (*$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared with the control group).

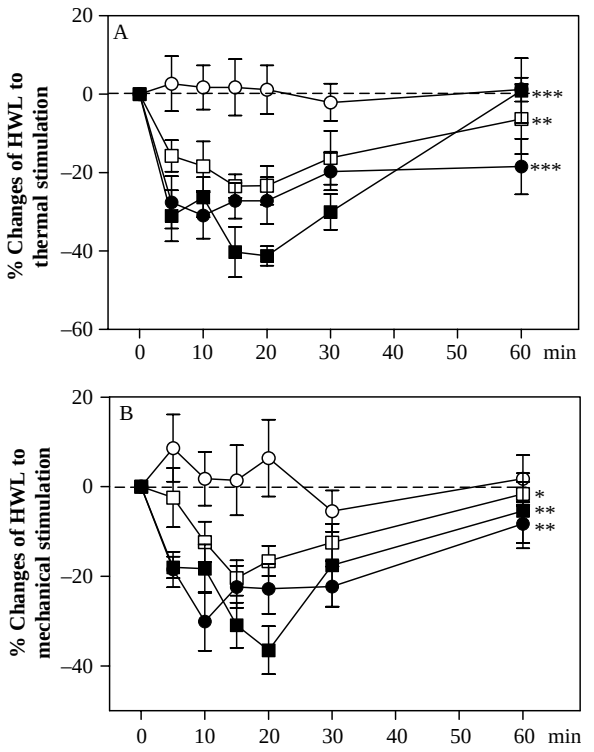

Fig. 7. Effects of intra-ARC injection of NPY28-36 on HWLs to thermal (A) and mechanical stimulation (B) in rats with inflammation. 1 μl of 0.9% saline (○); 0.1 nmol (□); 0.5 nmol (●); 1 nmol of NPY28-36 (■). The statistical difference between groups was determined by two-way ANOVA (*$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared with the control group).

of intact rats (NPY: $t=0.07$, $P=0.78$; NPY28-36: $t=0.60$, $P=0.46$; Fig. 8). Intra-ARC administration of 0.5 nmol of NPY or 1 nmol of NPY28-36 also did not change the performance of rats with inflammation (NPY: $t=0$, $P=1$; NPY28-36: $t=0.06$, $P=0.81$; Fig. 8).

3.8. Changes of Y1 receptor-like immunoreactivity in the ARC during inflammation

To investigate whether there is an influence of inflammation on the contents of NPY system in the ARC, intact rats ($n=4$) and rats with inflammation ($n=4$) were used for immunoreactive analysis. Y1 receptor-like immunoreactive neurons widely distribute in the ARC, matching the distribution of NPY-like immunoreactive fibers (Fig. 9).

Quantifications of the gray value of immunoreactivity of NPY and Y1 receptor were conducted using a 256-level gray scale. Four rats from each group and 6 sections from each rat were recorded and each point represents a gray value from one section (Fig. 10A-D). There were marked increases in Y1 receptor-like immunoreactivity in the bilateral ARC during inflammation (left, $t=34.82$, $P<0.01$; right, $t=26.97$, $P<0.01$; Fig. 10E) compared to intact ones, while there were no significant changes of NPY-like immunoreactivity (left, $t=0.74$, $P=0.42$; right, $t=0.71$, $P=0.45$; Fig. 10E).

4. Discussion

The results of present study demonstrate that administration of NPY to the ARC induced dose-dependent antinociception in intact rats and rats with inflammation.

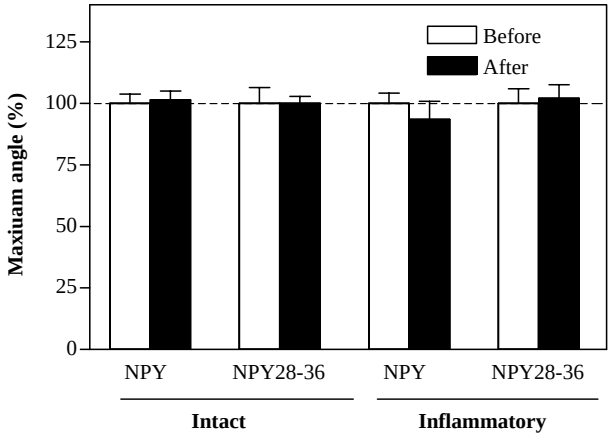

Fig. 8. Effects of intra-ARC administration of NPY or NPY28-36 on the motor function in the inclined plane test. Maximum angles were tested before and 20 min after intra-ARC injection of NPY or NPY28-36 in intact rats (NPY, $n=6$; NPY28-36, $n=6$) and rats with inflammation (NPY, $n=5$; NPY28-36, $n=5$). The statistical difference between groups was determined by Student two-tailed t-test.

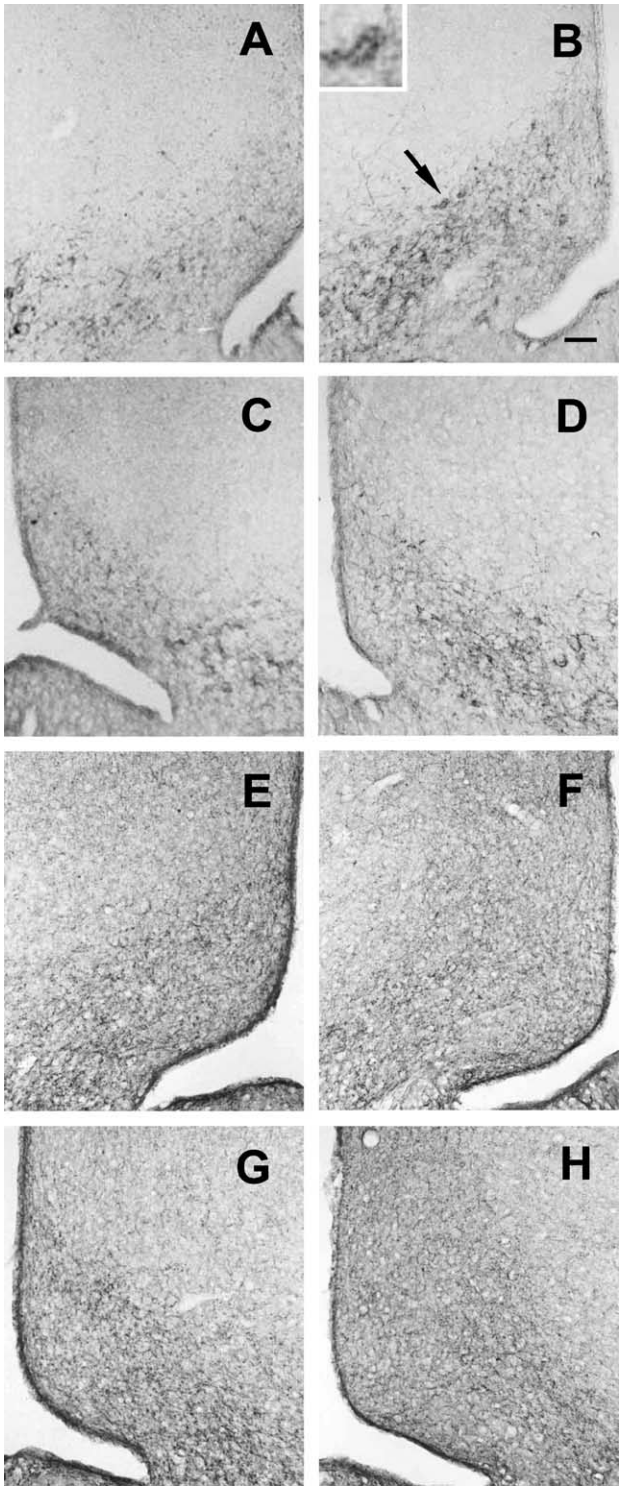

Fig. 9. Photomicrographs of Y1 receptor (A–D) and NPY (E–H) immunohiscochemistry in the ARC of intact rats and rats with inflammation. Y1-like immunoreactivity was analyzed in intact rats (A, C) and rats with inflammation (B, D). NPY-like immunoreactivity was analyzed in intact rats (E, G) and rats with inflammation (F, H). A,B,E,F show the left ARC, and C,D,G,H show the right ARC. The arrow points to the cells shown at higher magnification in the inset. Scale bar, 100 μm.

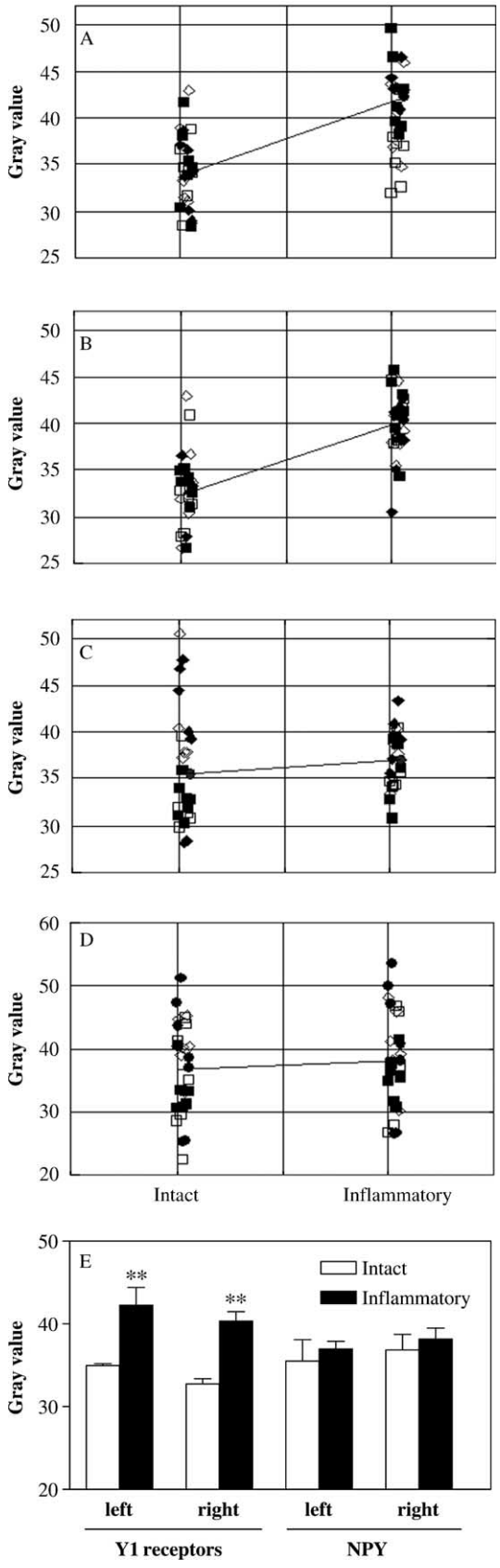

Fig. 10. Changes of Y1 receptor-like immunoreactivity in the ARC. The dynamic range of gray values of Y1 receptor (the left ARC: A; the right ARC: B) and NPY (the left ARC: C; the right ARC: D) in intact rats and rats with inflammation. B, The statistical average gray value of NPY and Y1 receptor in the ARC of rats with inflammation. The statistical difference between groups was determined by Student's two-tailed *t*-test (**$P<0.01$ compared with the control group).

Intra-ARC injection of either NPY antiserum or NPY28-36 enhanced the inflammation-induced hyperalgesia, suggesting an antinociceptive activity of tonic released NPY in the ARC of rats with inflammation. Moreover, there were up-regulations of Y1 receptor in the bilateral ARC during inflammation, which may contribute to the anti-nociceptive effect of NPY in the ARC.

4.1. Antinociceptive effects of NPY in the ARC of intact rats and rats with inflammation

It has been reported that NPY modulates nociceptive transmission in the spinal cord of intact rats (Duggan et al., 1991; Hua et al., 1991). In the brain, although the effect of intra-cerebroventricular administration of NPY on pain regulation seems to be both dose-dependent and test-dependent (Broqua et al., 1996; Mellado et al., 1996), the antinociceptive effect of NPY has been demonstrated in several important pain-related structures, such as the nucleus raphe magnus, PAG and nucleus accumbens (Li et al., 2002; Wang et al., 2001; Zhang et al., 2000). The present study demonstrate that intra-ARC administration of NPY produced dose-dependent antinociception, which was attenuated by subsequent intra-ARC administration of Y1 receptor antagonist NPY28-36. Furthermore, intra-ARC injection of Y2 receptor agonist NPY3-36 did not significantly alter the HWLs at the highest dose as used in the nociceptive test of NPY (0.5 nmol). The results indicate that the NPY-induced antinociception is mediated, at least partly, through activation of Y1 receptor, which agrees with our immunohistochemical analysis showing a high concentration of Y1 receptor in the ARC.

Besides the role of NPY in nociceptive modulation in intact rats, a role of NPY in inflammatory pain has been widely implicated (Wang et al., 2001; Xu et al., 1998). At spinal levels, behavioral studies indicate that intrathecal administration of NPY or its analogs produces thermal antinociception in models of transient pain (Hua et al., 1991) as well as decreases thermal hypersensitivity in rats with carrageenan- or CFA-induced inflammatory pain (Taiwo and Taylor, 2002). Furthermore, NPY also inhibits the ongoing inflammatory pain in rats with intra-plantar injection of formalin (Mahinda and Taylor, 2004). In the brain, the results of present study demonstrated that intra-ARC injection of NPY increased the HWLs in both thermal and mechanical tests in rats with carrageenan-induced inflammation.

NPY dose-dependently increased the nociceptive threshold in the ARC of intact rats and rats with inflammation. At lower doses, the D-R curve indicates

that NPY seems to play a less antinociceptive role to thermal stimulation after inflammation. However, there were no significant changes of NPY-induced antinociception to mechanical stimulation. At a high dose (0.5 nmol), NPY produced a greater antinociception in the Randall Selitto test during inflammation (Fig. 5). On the other hand, the analysis of ED50s showed that NPY had similar antinociceptive efficacy to thermal stimulation in both intact (ED50: 0.39 nmol) and inflammatory (ED50: 0.38 nmol) rats. However, the potency of NPY increased 4.3 fold in the paw pressure test after inflammation. This may be explained by the results of our neurochemical test, which showed an increased Y1 receptor after inflammation.

The complex changes of D-R relation after inflammation further indicate that the antinociceptive effect of NPY is dependent on special nociceptive tests, different pain models and varies doses (Mellado et al., 1996; Xu et al., 1998), which may be related to its diverse receptors (Rhim et al., 1997; Silva et al., 2002).

4.2. Antinociceptive effects of endogenous NPY in the ARC of rats with inflammation

Pain can be effectively controlled by various endogenous mechanisms (Machelska et al., 1998). Furthermore, high levels of endogenous NPY and Y1 receptors exist in the ARC. To unmask the effects of endogenous NPY in response to inflammatory pain, the NPY antiserum was used (Ossipov et al., 2002). We found that intra-ARC injection of NPY antiserum induced an enhanced hyperalgesia in rats with inflammation, suggesting that there is an inflammation-evoked tonic release of endogenous NPY in the ARC. However, the NPY antiserum had no effects in intact rats, implicating that the endogenous NPY may be silent or its effect is too low to be detected under normal condition.

Our results showed that there were significant increases of Y1 receptors in the bilateral ARC after inflammation. Moreover, intra-ARC injection of NPY28-36 produced no significant influences on the nociceptive threshold in intact rats, while intra-ARC administration of NPY28-36 alone reduced the HWLs of rats with inflammation. We also found that intra-ARC injection of NPY3-36 did not significantly influence the HWLs during inflammation. The results indicate that there may be a tonic release of endogenous NPY to activate Y1 receptor in the ARC, thereby playing a tonic anti-hyperalgesic role in rats with inflammation. Similarly, a recent study showed that intrathecal administration of Y1 receptor antagonist BIBO 3304 induces decreases in thermal hindpaw latencies ipsilaterally to the CFA injection (Tawio and Tayor, 2002). It is possible that the enhanced release of NPY and Y1-mediated inhibition on the spinal nociceptive transmission ultimately results in a compensatory, adaptive inhibition of thermal hypersensitivity in the setting of inflammation (Tawio and Tayor, 2002; Mahinda and Taylor, 2004).

In consistent with the endogenous antinociceptive effect NPY at spinal levels, the present study illustrates an involvement of tonically released NPY in the endogenous antinociceptive system at supraspinal levels.

4.3. Inflammation-induced increases in Y1 receptor in the ARC

Carrageenan-induced inflammation in the hindpaw has been shown to produce both thermal and mechanical hyperalgesia and activates genes leading to changes in the synthesis of various neuropeptides (Dubner and Ruda, 1992; Xu et al., 1999). It has been reported that unilateral paw inflammation elicits up-regulations in the transcription of NPY and Y1 receptor in the spinal cord (Ji et al., 1994). To evaluate the effects of inflammatory pain on the peptide system in the ARC, the immunohistochemical study was carried out. The results reveal that there were marked increases of Y1 receptor in the bilateral ARC, while no significant changes of NPY content were observed. This may be caused by a possible increased release of NPY in the ARC, or alternatively by the diffusion and/or rapid enzymatic breakdown of accumulated peptide (Wang et al., 2000). Taken together, the up-regulation of Y1 receptor, which is tonically stimulated by endogenous NPY, plays an important part in the NPY-induced antinociception in the ARC of rats with inflammation.

Six types of NPY receptors have been characterized (Silva et al., 2002). Recently, a role of Y1 receptor in nociceptive modulation has attracted much attention (Naveilhan et al., 2001; Wang et al., 2001; Zhang et al., 2000). It has been reported that Y1 receptor knockout mice not only exhibits hyperalgesic phenotype in several noxious tests, the analgesic response following intrathecal injection of NPY is also completely abolished (Naveilhan et al., 2001). Moreover, Taylor and coworker found that intrathecal injection of Y1 receptor antagonist could almost completely reverse the inhibitory effect of NPY on thermal hypersensitivity in CFA model and formalin-induced inflammatory pain test, while Y2 receptor antagonist has little effects (Mahinda and Taylor, 2004; Tawio and Taylor, 2002). Combined with the present study, these findings indicate a critical role of Y1 receptor in the NPY-induced antinociception in the central nervous system.

4.4. The Mechanisms of NPY-induced antinociception in the ARC

The mechanisms of the antinociceptive effect of NPY in the ARC with regard to nociceptive pathways remain unclear. The ARC is one of the most important brain regions where proopiomelanocortin (POMC), a precursor of melanocortins (MCs) is abundantly expressed (Mountjoy et al., 1994). It has been proved that NPY-containing nerve terminals synaptically contact with POMC-positive neurons expressing of Y1 receptor (Broberger et al., 1997; Cowley

et al., 2001). Further evidence shows that in either prepared slices or dissociated neurons, NPY induces inhibitory effects on POMC-ergic neurons through activation of Y1 receptor in the ARC (Roseberry et al., 2004).

A major projection of POMC-containing neurons in the ARC is directly to the PAG (Chen et al., 1992; Yoshida and Taniguchi, 1988), where both MCs and MCs receptors have been found (Palkovits and Eskay, 1987; Xia et al., 1995). Previous studies demonstrated that intrathecal administration of MCs receptors antagonist alleviates allodynia in mononeuropathic rats, while its agonist has the opposite effect (Starowicz et al., 2002; Vrinten et al., 2000, 2001). More importantly, it has been suggested that central administration of MCs induces hyperalgesia in various nociceptive models (Bertolini et al., 1979; Sandman and Kastin, 1981; Williams et al., 1986). It is possible that the MCs released in the PAG by the POMC-containing neurons in the ARC may contribute to the hyperalgesia of pathological pain. Thus, NPY probably acts on Y1 receptor to inhibit the excitability of the POMC-ergic neurons, reducing the release of MCs in the PAG to produce antinociception.

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