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Y1 RECEPTOR KNOCKOUT INCREASES NOCICEPTION AND PREVENTS THE ANTI-ALLODYNIC ACTIONS OF NPY

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Abstract

Recent pharmacological studies in our laboratory suggest that the spinal neuropeptide Y (NPY) Y1 receptor contributes to pain inhibition and to the analgesic effects of NPY. To rule out off-target effects, the present study used Y1 receptor deficient ($-/-$) mice to further explore the contribution of Y1 receptors to pain modulation. Y1 $-/-$ mice exhibited reduced latency in the hotplate test of acute pain and a longer-lasting heat allodynia in the CFA model of inflammatory pain. Y1 deletion did not change CFA-induced inflammation. Upon targeting the spinal NPY systems with intrathecal drug delivery, NPY reduced tactile and heat allodynia in the CFA model as well as the partial sciatic nerve ligation model of neuropathic pain. Importantly, we show for the first time that NPY does not exert these anti-allodynic effects in Y1 $-/-$ mice. Furthermore, in nerve-injured CD1 mice, concomitant injection of the potent Y1 antagonist BIBO 3304 prevented the anti-allodynic actions of NPY. Neither NPY nor BIBO3304 altered performance on the rotarod test, arguing against an indirect effect of motor function. We conclude that the Y1 receptor contributes to pain inhibition and to the analgesic effects of NPY.

Keywords

Allodynia; Hyperalgesia; Pain; Mouse; Neuropathic; Inflammation

INTRODUCTION

Both neuropeptide Y and the NPY Y1 receptor are highly expressed at key sites of pain transmission, including lamina II of the spinal cord dorsal horn [1,2]. These anatomical findings position the NPY system as a key player in pain control. In our previous studies, we targeted this system with intrathecal delivery of NPY receptor ligands. NPY dose-dependently reduced behavioral signs of inflammatory and neuropathic pain [3–5]. In the rat, these anti-allodynic actions could be reversed with the NPY Y1 receptor antagonist, BIBO3304 [3,5]. When administered by itself, BIBO3304 slightly enhanced injury induced allodynia. Although this

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compound is reportedly quite selective and potent at Y1, our study could not rule out off-target effects. Fortunately, the development of NPY receptor knockout mice has allowed us to further explore the contribution of Y1 receptors to pain modulation [H. Herzog, 9TH international NPY meeting]. Thus, to test the hypothesis that the Y1 receptor contributes to pain inhibition and to the analgesic effects of NPY, we evaluated behavioral responses in Y1 receptor-deficient mice.

METHODS

Animals

Either male CD-1 mice (Charles River Laboratories, Wilmington, MA) or male NPY Y1 receptor-deficient mice ($-/-$), back-crossed for more than 6 generations into a C57BL/6 background [6], were used at 2–5 months of age. Genotype of heterozygous progeny was determined by PCR, yielding ($-/-$) and wildtype ($+/+$) controls. Heterozygous progeny were not used in the present study. Mice were housed in individual cages in a temperature controlled room ($23 \pm 1^\circ\text{C}$, humidity $50 \pm 10\%$) on a 12-hour light/dark cycle (6am/6pm), and were given food and water *ad libitum*. All animal use protocols were approved by the Institutional Animal Care and Use Committee of the University of Missouri, Kansas City and Tulane University.

Behavioral pain tests

Hot plate test of transient pain—Y1-wildtype or Y1-deficient mice were acclimatized to a hot plate (Columbus instruments, Columbus, Ohio), pre-heated to 30°C , two times on each of two days prior to testing. With the experimenter blind to strain, mice were tested on a hotplate set to 52°C , then 55°C , and then 58°C . Response latency was determined as the time taken to lick a hind paw or jump. Triplicate measurements, with at least 10 min between each trial, were averaged.

Unilateral paw withdrawal latency to heat—Mice were acclimated to a transparent plexiglass enclosures for a 2-h period. The cages rested upon a thermal paw stimulator system (Department of Anesthesiology, University of California, San Diego, CA, USA; [7]), modified from the original design [8]. The device consisted of a glass surface maintained at a constant temperature of 25°C . On the day of testing, after an additional hr of acclimation, a radiant heat beam was activated and paw withdrawal latency (PWL) was obtained from each hindpaw. The first two measurements were discarded (they tended to be unusually high and variable), and the 4 subsequent measurements were averaged. Voltage intensity was adjusted such that latency in control mice was 12 ± 2 s. If the animal did not respond within 20 s, the heat was discontinued to prevent tissue damage.

Tactile threshold—Tactile threshold was assessed with an incremental series of 8 von Frey filaments of logarithmic stiffness (Stoelting, Inc.; approximately 0.008 - 6.0 g). The 50% withdrawal threshold was determined using the up-down method of Dixon, modified by Chaplan et al [9]. First, an intermediate von Frey hair (0.16g) was applied perpendicular to the hindpaw surface with sufficient force to cause a slight bending of the filament. In case of a positive response (rapid withdrawal of the paw within 3 sec), the next smaller filament was tested. In case of a negative response, the next larger filament was tested

CFA-evoked cutaneous thermal hyperalgesia

Following baseline thermal or tactile testing, 5–10 μL of complete Freund's adjuvant (CFA = killed mycobacterium butyricum suspended in 10 mg/ml mineral oil, Sigma) was injected into the ventral midplantar right hindpaw. In one experiment (Fig 2), we evaluated heat PWL and paw thickness (using a microcaliper, Mitutoyo) at 1, 2, 3 and 7 days after CFA. At least two

measurements were made at every time point. In the other experiment (Fig 3), we evaluated tactile threshold 30, 90, and 150 min after intrathecal injection of NPY.

Partial Sciatic Nerve Injury

Mice were anesthetized with either ketamine/xylazine (100/15 mg/kg) or isoflurane (5% induction, then 1.5% maintenance in oxygen). As previously described [10], an incision was made in the skin at the high-thigh level, and the sciatic nerve was dissected from surrounding connective tissue near the trochanter, just distal to the branching point of the posterior biceps semitendinosus nerve. Using 9-0 silk suture, $\frac{1}{3}$ to $\frac{1}{2}$ the diameter of the nerve was tightly ligated, followed by wound closure with 4-0 silk suture. After a 7d recovery period, mice were tested for heat PWL at the hind paw on the same (ipsilateral) or opposite (contralateral) side relative to nerve injury.

Intrathecal Drug Administration

To target the spinal cord in awake behaving rodents, pharmacological studies typically utilize the intrathecal route of administration. As previously described [11], drugs or vehicle were injected in a volume of 5 μ l using a 30g \times $\frac{1}{2}$ -inch needles (B-D Yale, Rutherford, NJ) connected to a 10 μ l luer tip syringe (Hamilton, Reno, NV). With the spinal column firmly stabilized at the iliac crest, the needle was inserted dorsally between the spinous and transverse processes of the L5 and L6 vertebrae, angled, and advanced. The injection was made after observation of a tailflick response or hindlimb retraction.

Behavioral test of ataxia

Mice were placed on a rotating bar (diameter = 3.5 cm, Economex, Columbus Instruments, Columbus, OH) that accelerated logarithmically from approximately 4 rpm to 24 rpm in 3 min. Performance duration was recorded when the animal, unable to stay on the rotarod, fell a short distance, tripped a plate, and automatically stopped a timer. Mice were trained for a minimum of 3 d prior to testing. Mice that repeatedly fell off the bar within 45 s or remained on the bar over 5 min were excluded from further testing. Before injection and 30 and 60 min after injection, 4 measurements were recorded and averaged.

Materials

All drugs were prepared fresh daily and stored on ice before use. Human NPY was obtained from Anaspec (San Jose, CA). The Y1 receptor antagonists BIBO 3304 was provided by H. Doods (Boehringer Ingeleim, Biberach, Germany). Morphine was obtained from NIDA (Drug Supply Program). Xylazine was obtained from Boehringer Ingelheim Vetmedica, Inc. (St. Joseph, MO) and ketamine from Fort Dodge Animal Health (Fort Dodge, IA).

Statistical analyses

We used GraphPad Prism 4 (GraphPad Software, Inc. San Diego, CA) and Systat 11 software to conduct two-way analysis of variance (ANOVA), with Strain or Drug as the between-subjects factor and either hotplate temperature as the within-subjects factor or time as the repeated measure. When F values were significant ($p < 0.05$), Bonferroni post hoc test was used to determine differences between two groups. All data are presented as mean \pm SEM.

RESULTS

As reported previously [6][Higuchi, 9th International NPY meeting], Y1 knockout mice appeared grossly normal, with slightly elevated body weight (wildtype = 28.7 \pm 0.5g, knockout = 31.2 \pm 1.1g; $p = 0.05$ by t-test, $n = 12$).

Y1 receptor deletion increases transient heat pain

The hotplate test is a standard method to assess acute pain in rodents. Figure 1 illustrates that Y1 receptor-deficient mice exhibit reduced latency (e.g. increased nociception) in the hotplate test ($F(1,72)=13$, $P<0.0005$).

Y1 receptor deletion increases inflammatory pain

The NPY system is integrally linked to the modulation of inflammation [Marks, 9th International NPY meeting]. To test the hypothesis that endogenous Y1 tonically inhibits inflammatory pain, we evaluated the time course of CFA-induced thermal allodynia in germline Y1 knockouts and their littermate wild-type controls. To avoid a “floor effect”, below which further decreases in heat latency could not be observed, we used a low dose of CFA (5 μ l). Figure 2A illustrates that this dose of CFA evoked heat allodynia that lasted just a single day in wild-type mice. By contrast, CFA produced a longer-lasting hyperalgesia in Y1-deficient mice ($F(1,70)=31$, $p<0.0001$).

Previous studies suggest that the Y1 receptor contributes to inflammation [12]. Because inflammation is intricately associated with inflammatory pain, we evaluated paw thickness, a gross measure of edema. As illustrated in Fig 2B, Y1 deletion did not change the paw edema associated with CFA injection.

Y1 receptor deletion reduces NPY inhibition of inflammatory and neuropathic pain

While previous studies indicate that Y1 receptors contribute to NPY analgesia in the tailflick model of acute nociception [12], rigorous studies have not been performed in models of chronic pain. To address this question, we evaluated the effects of intrathecal NPY in wildtype and Y1 knockout mice in models of inflammatory and neuropathic pain. Figs 3A–B illustrates that a greater volume of CFA (10 μ g i.pl.) produced a robust tactile allodynia in Y1 wild-type and knockout mice. Intrathecal injection of saline did not change tactile threshold. Importantly, intrathecal NPY (10 μ g) reduced tactile allodynia on the side of inflammation in wild-type but not Y1 knockout mice. Neither saline nor NPY changed tactile threshold at the contralateral side. Fig 3C illustrates similar results in the pSNL model of neuropathic pain: NPY reduced heat allodynia on the side of nerve injury to a greater degree in wild-type than Y1 knockout mice ($F(1,85)=14$, $p<0.0005$). Neither vehicle nor NPY produced a change in heat response latency at the contralateral side.

We previously reported that, in the rat, intrathecal NPY does not disrupt motor coordination [3]. To extend this to the mouse, and thus rule out the possibility that indirect motor effects mediated the anti-allodynic effects of NPY, we used the rotarod test of ataxia. As illustrated in Fig 3D, NPY (10 μ g), as compared to vehicle, did not produce ataxia. By contrast, the positive control (morphine, 1.6 μ g) reduced time spent on the accelerating rotarod.

Y1 receptor antagonism prevents NPY inhibition of neuropathic pain

Our conventional knockout strategy lacks temporal specificity and is subject to compensatory changes during development. Therefore, to further test the hypothesis that the Y1 receptor contributes to the anti-allodynic effect of NPY, we established heat allodynia in the pSNL model, and then co-administered the Y1-selective receptor antagonist BIBO3304 with either NPY or vehicle. As illustrated in Fig 4, BIBO3304 prevented the anti-allodynic effect of NPY on the side of nerve injury ($F(1,60)=7.6$, $p<0.01$). When administered in the absence of NPY, BIBO3304 did not significantly change PWL ($p<0.05$, data not shown). BIBO3304 did not change performance on the accelerating rotarod ($p<0.05$, data not shown).

DISCUSSION

The Y1 receptor system exerts intrinsic inhibitory pain control

Acute Pain—Our current results indicate that Y1-deficiency increases acute nociception. This confirms previous studies that used an alternative strain of Y1 deletion mutant mice [12]. In addition to reduced hotplate latency, these mice exhibited exaggerated behavioral signs of acute nociception following the administration of noxious formalin, acetic acid, or magnesium sulfate [12]. Taken together, these results suggest that the Y1 receptor system exerts tonic inhibitory control over acute pain.

Due to the germ-line nature of the Y1 deletion, our studies do not discriminate between different locations Y1 inhibition. As indicated by pharmacological studies, these locations include spinal and supraspinal sites. For example, we and others have shown that intrathecal administration of NPY receptor agonists reduces behavioral responsiveness to noxious heat in un-anesthetized rodents [3,12,13].

What is the source of endogenous ligand for intrinsic spinal Y1 inhibition of acute pain? Several observations argue against a contribution of NPY produced by cells of the dorsal root ganglia (DRG) and released from their central terminals. First, NPY immunoreactivity and mRNA is virtually absent in DRG neurons, even during inflammation [1,2,14,15]. Furthermore, the spontaneous release of NPY is not significantly altered by electrical stimulation of peripheral nerves [16]. Therefore, a more likely source of NPY are intrinsic interneurons of lamina I–III [1,17]. Many of these NPY-containing interneurons also express GABA and form synaptic contacts with pain-related neurons in the dorsal horn [18].

In the brain, the Y1 receptor is expressed in multiple brain regions thought to modulate pain, including the midline rostral ventral medulla [19–21]. In recent years, targeting of such brain regions with an expanding use of intraparenchymal administration strategies has greatly contributed to our understanding of NPY analgesic action. Following the microinjection of NPY into the RVM or other pain modulatory areas such as the periaqueductal gray, nucleus accumbens, or acute nucleus of the hypothalamus, withdrawal reflexes to noxious heat or tactile stimuli are decreased [22–25]. Such effects may be mediated by CNS Y1 receptors, since BIBO3304 reversed that anti-allodynic effects of intra-RVM injection of NPY in two different models of peripheral neuropathic pain [26].

Inflammatory Pain—Previous studies have reported that Y1 deletion mutant mice display exaggerated behavioral responses to the intraplantar administration of carrageenan (a model of acute inflammatory pain) [12]. More relevant to clinical pain, however, are models of persistent inflammatory pain, such as that induced by CFA. Our current results indicate that Y1 deficiency extends the duration of inflammatory pain in the CFA model. Y1 deletion did not change the inflammatory response to CFA; therefore, increased inflammatory pain is not a consequence of strain differences in the time course of inflammation. Together with our previous finding that intrathecal administration of BIBO3304 increased thermal hypersensitivity to CFA [3], we conclude that Y1 receptors in the dorsal horn contribute to the homeostatic control of inflammatory hyperalgesia.

NPY acts at Y1 receptors in the spinal cord to inhibit allodynia

Inflammatory Pain—The present results show for the first time that Y1 deletion prevents the ability of intrathecal NPY to reduce inflammation-induced tactile allodynia, indicating that the Y1 receptor mediates the anti-allodynic actions of NPY during inflammatory pain. Reinforcing this conclusion is our previous finding that BIBO3304 attenuated the inhibitory effect of NPY not only in the CFA model, but also in the intraplantar formalin model of acute

inflammatory pain [3,27]. Interestingly, CFA increases the expression of Y1 receptors in the dorsal horn [2], leading us to speculate that inflammation increases the tonic NPY-Y1 inhibition of spinal nociceptive transmission, thus contributing to anti-allodynic effects of NPY.

Neuropathic pain—As with inflammatory pain, we show for the first time that Y1 deletion reduces the anti-allodynic effects of NPY in the partial sciatic nerve ligation model of neuropathic pain. Furthermore, we show that the Y1 receptor antagonism prevents NPY anti-allodynia. In addition to inhibition of allodynia, we have also reported that intrathecal NPY reduced peripheral nerve injury-induced expression of Fos, a marker of cellular activation in the dorsal horn. BIBO3304 blocked this effect of NPY, indicating that the Y1 receptor is negatively linked to spinal neuron activation[5]. In summary, with the caveat that Fos labels both excitatory and inhibitory neurons, we conclude that the Y1 receptor is necessary for the anti-allodynic effects of intrathecal NPY.

Mechanism of pharmacological NPY-Y1 analgesia—By what neurochemical mechanism does NPY act at Y1 receptors to inhibit pain? As previously reviewed, NPY activation of Y2 receptors on presynaptic terminals of primary afferent neurons is thought to contribute to intrathecal NPY analgesia [4]. This does not rule out a similar contribution of the Y1 receptor, which substantially co-localizes with selective markers of primary afferent neurons, CGRP and TRPV1. As such, the Y1 receptor is poised to modulate the release of pronociceptive neurotransmitters [28,29]. Indeed, the Y1 antagonist BIBP3226 reverses the inhibitory effect of NPY on CGRP release in spinal cord slices [29]. In addition to such presynaptic mechanisms, we believe that NPY acts postsynaptically at Y1 receptors in lamina II of the dorsal horn. Such actions are via complex neurophysiological mechanisms that may involve inhibition of GABA release than then inhibits molecular (Fos activation) and behavioral (heat hypersensitivity) signs of inflammatory pain [4].

In summary, we found that Y1 deletion increases acute pain and decreases NPY analgesia. We suggest that NPY receptors, particularly at spinal sites of pain modulation, represent important targets for the therapeutic development of NPY agonists for the treatment of chronic pain.

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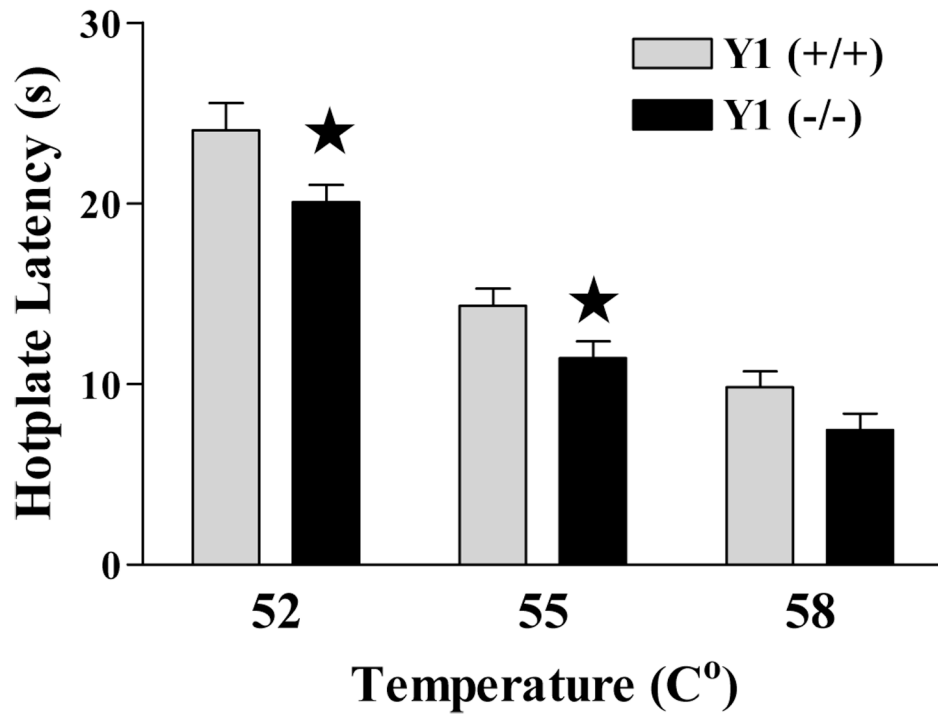


Figure 1. Y1 receptor deletion increases transient heat pain
Hotplate latency in wildtype (+/+) and Y1-deficient (-/-) mice. n=13 per group. Values represent mean \pm SEM. *p<0.05.

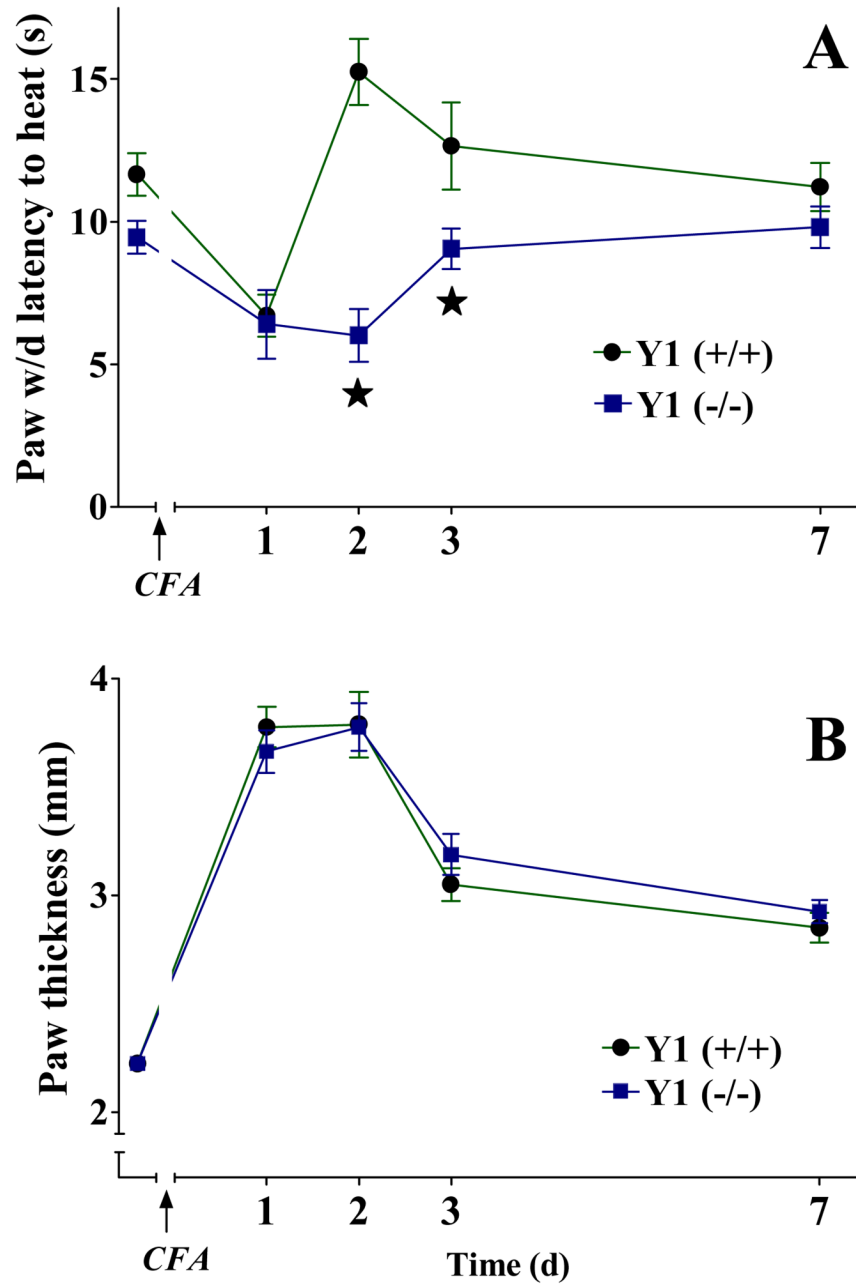


Figure 2. Y1 receptor deletion increases inflammatory pain

Time course of heat hyperalgesia (A) and edema (B) after the intraplantar injection of CFA in wildtype (+/+) and Y1-deficient (-/-) mice. n=8 per group. Values represent mean ± SEM. *p<0.05.

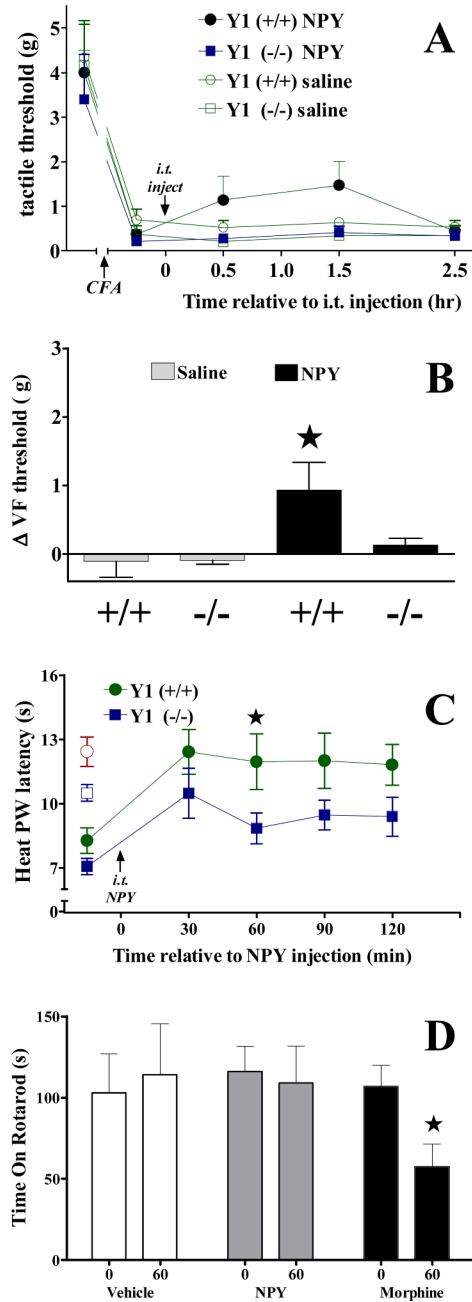


Figure 3. Y1 receptor deletion reduces NPY inhibition of allodynia

Panel A (inflammatory pain): Line graphs depicting the time course of von Frey threshold in Y1 wild-type (+/+) and Y1 knockout (-/-) mice before and one day after the intraplantar injection of CFA, and then after intrathecal (i.t.) injection of saline or NPY (10 μg) at time t=0. n=3–5 per group. **Panel B:** the data of Panel A plotted as the difference between pre-injection and post-injection (30–90 min time points) values. **Panel C (neuropathic pain):** The time course of hindpaw withdrawal latency to heat in wild-type and knockout mice after i.t. NPY (10 μg). Prior to i.t. injection, latency was reduced on the side ipsilateral to partial sciatic nerve ligation (closed symbols) but not the contralateral side of wildtype (open circles) or knockout mice (open squares). n=9–10 per group. **Panel D (ataxia):** Histograms depicting time spent on

an accelerating rotarod in uninjured CD1 mice after the intrathecal injection of vehicle, 10 μ g NPY, or the positive control 1.6 μ g morphine. Values represent mean \pm SEM. * p <0.05.

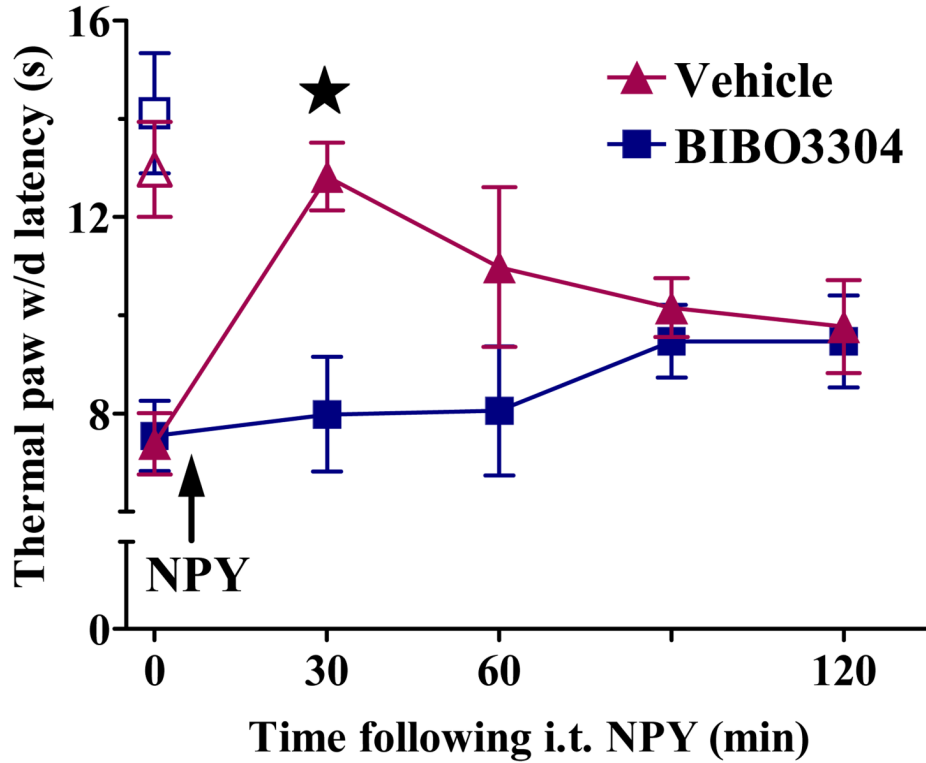


Figure 4. Y1 receptor antagonism prevents NPY inhibition of neuropathic pain

The time course of hindpaw withdrawal latency to heat in CD1 mice after i.t. NPY (10 μ g) when co-administered with vehicle or the Y1 antagonist, BIBO3304 (10 μ g). Prior to injection, latency was reduced on the side ipsilateral to partial sciatic nerve ligation (closed symbols) but not the contralateral side of mice co-injected with dH₂O (open triangles) or BIBO (open squares). Values represent mean \pm SEM. n=6 per group. *p<0.05.