

Pharmacologic Characterization in the Rat of a Potent Analgesic Lacking Respiratory Depression, IBNtxA

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ABSTRACT

IBNtxA (3'-iodobenzoyl-6 β -naltrexamide) is a potent analgesic in mice lacking many traditional opioid side effects. In mice, it displays no respiratory depression, does not produce physical dependence with chronic administration, and shows no cross-tolerance to morphine. It has limited effects on gastrointestinal transit and shows no reward behavior. Biochemical studies indicate its actions are mediated through a set of μ -opioid receptor clone MOR-1 splice variants associated with exon 11 that lack exon 1 and contain only six transmembrane domains. Like the mouse and human, rats express exon 11-associated splice variants that also contain only six transmembrane domains, raising the question of whether IBNtxA would have a

similar pharmacologic profile in rats. When given systemically, IBNtxA is a potent analgesic in rats, with an ED₅₀ value of 0.89 mg/kg s.c., approximately 4-fold more potent than morphine. It shows no analgesic cross-tolerance in morphine-pelleted rats. IBNtxA displays no respiratory depression as measured by blood oxygen saturation. In contrast, oximetry shows that an equianalgesic dose of morphine lowers blood oxygen saturation values by 30%. IBNtxA binding is present in a number of brain regions, with the thalamus standing out with very high levels and the cerebellum with low levels. As in mice, IBNtxA is a potent analgesic in rats with a favorable pharmacologic profile and reduced side effects.

Introduction

Opiates have long been acknowledged as essential to treating pain clinically, but with a risk of diversion and addiction. Efforts to generate opioids that lack side effects and abuse potential go back over a century (Pasternak and Pan, 2013). Although many important analgesics and antagonists were developed, these agents did not dissociate pain relief from side effects. Current evidence suggests that this is possible, starting with the pharmacologic dissociation of morphine analgesia from respiratory depression and constipation by the antagonists naloxonazine and naloxazone (Pasternak et al., 1980; Wolozin and Pasternak, 1981; Hahn et al., 1982; Hahn and Pasternak, 1982; Ling et al., 1983, 1984, 1985, 1986).

After the cloning of the μ -opioid receptor (MOR-1) and its gene *Oprm1*, our group and others isolated and characterized

a vast array of μ -receptor splice variants (for a review, see Pasternak and Pan, 2013). These variants fall into three major categories. The first includes the full-length variants containing the traditional μ -receptor variants with seven transmembrane (TM) domains that differ by 3'-splicing of the C terminus, leading to the replacement of exon 4 and its terminal 12 amino acids with a range of various combinations of alternative exons yielding from 1 to over 80 alternative unique amino acid sequences in their place (Bare et al., 1994; Zimprich et al., 1994, 1995; Pan et al., 1999, 2005; Pasternak et al., 2004). Another set of variants contains a single TM domain corresponding to TM1 in the traditional receptors (Du et al., 1997; Xu et al., 2013). Although this set does not directly bind opioids, it exerts significant pharmacologic actions through its chaperone-like function, which stabilizes the full-length variants and increases their expression levels in both tissue culture models and in vivo (Xu et al., 2013).

The final set of splice variants are associated with exon 11 and contain only six transmembrane (6TM) domains, lacking TM1 present in the traditional full-length receptors (Pan et al., 2001; Xu et al., 2011). Initially thought to be of little significance, they have proven very important in understanding μ -opioid pharmacology, as revealed by a series of knockout

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ABBREVIATIONS: CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂; DOR-1, δ -type opioid receptor; DPDPE, D-Pen², D-Pen⁵-enkephalin; IBNtxA, 3'-iodobenzoyl-6 β -naltrexamide; KO, knockout; KOR-1, κ -type opioid receptor; MOR-1, μ -opioid receptor; NalBzoH, naloxone benzoylhydrazine; norBNI, norbinaltorphimine; TM, transmembrane domain; U50,488H, (*trans*)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide.

(KO) mice. A complete μ KO (E1/E11 KO) in which both exon 1 and exon 11 are disrupted is insensitive to all the μ drugs tested, confirming the importance of the *Oprm1* gene in their actions (Y. X. Pan and G. W. Pasternak, unpublished data). An exon 1 MOR-1 KO mouse generated by Pintar eliminates the full-length 7TM variants while still expressing the truncated 6TM ones (Schuller et al., 1999). Conversely, an exon 11 MOR-1 KO mouse lacks the 6TM variants while still expressing the full-length ones (Pan et al., 2009; Majumdar et al., 2011b; Pasternak and Pan, 2013). As expected, loss of the traditional receptors in the exon 1 KO mouse eliminates morphine analgesia. In contrast, morphine and methadone retain full analgesic activity in the exon 11 KO animals. However, the analgesic activity of a number of other opioids with μ actions are significantly attenuated or completely lost in the exon 11 KO mice, including levorphanol, butorphanol, buprenorphine, and nalbuphine (Pan et al., 2009; Majumdar et al., 2011b, 2012; Pasternak and Pan, 2013). Thus, these KO models indicate the existence of two classes of μ opioids, the traditional morphinelike ones acting through full-length receptors and atypical ones working through the truncated 6TM splice variants.

Recently, we synthesized a highly potent analgesic, IBNtxA (3'-iodobenzoyl-6 β -naltrexamide), with a very unusual pharmacologic profile (Majumdar et al., 2011b, 2012; Pasternak and Pan, 2013). Despite its high analgesic potency in mice, it exhibits no respiratory depression at doses up to 5-fold greater than its analgesic ED₅₀, has little effect on gastrointestinal transit, and fails to show either reward or aversive behavior in a conditioned place preference paradigm. When administered chronically, it produces no physical dependence and shows no cross-tolerance to morphine. IBNtxA analgesia is fully retained in the Pintar exon 1 KO, clearly distinguishing its mechanisms from those of morphine. The analgesia was not the result of cross-labeling traditional δ or κ_1 receptors because IBNtxA still retained full analgesic activity in a triple KO mouse lacking all traditional opioid receptors generated by crossing Pintar's exon 1 MOR-1 KO with δ -type opioid receptor (DOR-1) KO and κ -type opioid receptor (KOR-1) KO animals.

Further studies using [¹²⁵I]IBNtxA in the mouse identified a molecular target for the drug that depends on the truncated 6TM exon 11-associated variants. [¹²⁵I]IBNtxA labels this site in the mouse brain with subnanomolar affinity and shows a similar affinity and B_{\max} value in the triple KO mice. Thus, IBNtxA acts through a novel opioid receptor target of action with a favorable side-effect profile in mice. Like the mouse, rats and humans also express truncated 6TM receptors associated with exon 11 (Pasternak et al., 2004; Xu et al., 2007; Pasternak and Pan, 2013). In this study, we examine the pharmacology of IBNtxA in the rat.

Materials and Methods

IBNtxA and [¹²⁵I]IBNtxA were synthesized and their structures confirmed as previously described elsewhere (Majumdar et al., 2011a,b, 2012). Opioids were obtained from the Research Technology Branch of the National Institutes of Health National Institute on Drug Abuse (Rockville, MD) or Tocris Bioscience (Bristol, UK). Miscellaneous chemicals and buffers were purchased from Sigma-Aldrich (St. Louis, MO).

Male Sprague Dawley rats were obtained from Charles River Laboratories (Hollister, CA). All rats were maintained on a 12-hour

light/dark cycle with Purina rodent chow Diet 5053 and water available ad libitum, and they were housed in groups of two until testing. All animal studies were approved by the Institutional Animal Care and Use Committee of the Memorial Sloan-Kettering Cancer Center.

Binding Studies. [¹²⁵I]IBNtxA binding assays were performed in brain membrane homogenates prepared as previously described elsewhere at a concentration of 0.5–1.0 mg protein/ml (Clark et al., 1988, 1989; Majumdar et al., 2011b). To prevent binding to traditional opioid receptors, binding was performed in the presence of μ (CTAP [D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂]), κ_1 (U50,488H [(trans)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide]), and δ (DPDPE [D-Pen²,D-Pen⁵-enkephalin]) blockers at a final concentration of 250 nM each. Nonspecific binding was determined in the presence of levallorphan (1 μ M), and only specific binding is reported. Binding reached equilibrium at 25°C after 90 minutes (data not shown). Binding was performed for 90 minutes at 25°C using 0.5–1.0 ml of homogenate. Glass-fiber filters were soaked in 0.5% polyethyleneimine for at least 15 minutes before filtration to minimize the nonspecific binding to the filters.

K_D , B_{\max} , and IC₅₀ were fit by nonlinear regression using GraphPad Prism (GraphPad Software, La Jolla, CA). Each IC₅₀ value was converted to give an apparent

$$K_i = IC_{50}/(1 + ([L]/K_D)),$$

as previously reported elsewhere (Cheng and Prusoff, 1973). Hill slopes were calculated using a linear regression of a probit transform from competition experiments using values from 15–85% inhibition (Litchfield and Wilcoxon, 1949). All experiments were replicated at least three times.

For regional binding assays, rat brains were rapidly removed and placed in ice-cold phosphate-buffered saline (50 mM; pH 7.4) and dissected into the designated regions. Membrane preparations were made and binding performed with [¹²⁵I]IBNtxA (0.1 nM) in the presence of CTAP, DPDPE, and U50,488 (250 nM each) to block traditional μ , δ , and κ binding. Binding was normalized to the whole brain. Results are the mean \pm S.E.M. of three independent experiments. Regional binding was compared using a one-way analysis of variance followed by a Bonferroni multiple-comparison test.

Analgesia Assay. ED₅₀ values were determined using a cumulative dose-response approach in which rats were tested with escalating doses of IBNtxA 30 minutes after the prior subcutaneous injection using a radiant heat tail-flick paradigm as previously published elsewhere (Ling et al., 1985; Majumdar et al., 2011b). A maximal latency of 10 seconds was used to minimize tissue damage. ED₅₀ values were determined by nonlinear regression analysis (GraphPad Prism). Results are expressed as the percentage of maximum possible effect (%MPE) for each individual rat according to the equation %MPE = [(Observed latency – Baseline latency)/(Maximal latency – Baseline latency)]. To assess for cross-tolerance between IBNtxA and morphine, rats were made tolerant to morphine by the subcutaneous implantation of three 75-mg pellets of morphine freebase each (total dose 225 mg) or placebo pellets under isoflurane anesthesia (Ling et al., 1984).

Respiratory Function Assay. The effect of the drugs on respiration was assessed using oximetry. Blood oxygen saturations were assessed in awake, freely moving rats (175–225 g) with the MouseOx pulse oximeter system (Starr Life Sciences, Holliston, MA). A 5-second average oxygen saturation value was measured every 5 minutes. Each animal was habituated to the device for 30 minutes to establish a baseline before injection.

Results

In Vivo Actions of IBNtxA. IBNtxA is a potent analgesic in mice in the radiant heat tail-flick assay. Similarly, it was an

effective analgesic in rats (Fig. 1). Time action studies revealed a peak effect at 30 minutes after subcutaneous administration (not shown), similar to most opioids. At peak effect, IBNtxA had an analgesic ED₅₀ of 0.89 mg/kg (95% confidence interval, 0.69–1.2) (Fig. 1A). This was approximately 2-fold less potent than in mice (ED₅₀ = 0.48 ± 0.05 mg/kg) (Majumdar et al., 2011b) and approximately 4-fold more potent than morphine.

Morphine actions are readily reversed by the antagonist naloxone. The opioid antagonist levallorphan potentially reverses IBNtxA in mice. Similarly, levallorphan effectively reversed IBNtxA in the rat (Fig. 1B). We next determined the sensitivity of IBNtxA analgesia to naloxone. First, we compared the ability of naloxone to reverse morphine and IBNtxA analgesia (Fig. 1C). At 1 mg/kg, naloxone fully reversed morphine's response. Naloxone also effectively antagonized the actions of IBNtxA at both 1 and 5 mg/kg s.c., confirming the opioid nature of the response. However, IBNtxA analgesia was far less sensitive to naloxone than morphine (Fig. 1D). A lower naloxone dose (0.1 mg/kg s.c.) still fully reversed morphine analgesia but had no effect on IBNtxA activity. This relative insensitivity of IBNtxA analgesia to naloxone is similar to that previously observed in mice (Majumdar et al., 2011b).

Cross-tolerance is often used to demonstrate a common mechanism of action. Traditionally, μ opioids show cross-tolerance to morphine, although this is often incomplete (for a review, see Pasternak and Pan, 2013). One standard paradigm is to implant rats with pellets of free-base morphine, resulting in its slow release over days (Cicero and Meyer, 1973; Yoburn et al., 1985). In this approach, rats are implanted with three morphine free-base pellets each (225 mg total), and the rats are assessed 3 days later. This paradigm leads to marked morphine tolerance and dependence. For the first few hours, all rats displayed full analgesia. When they were assessed after 3 days, no residual analgesia was detected despite the continued presence of the morphine pellets. The rats were both tolerant and dependent. When challenged with acute doses, the morphine-pelleted animals displayed a 6-fold decrease in morphine analgesia ($P < 0.001$), with the ED₅₀ increasing from 3.3 to 20.5 mg/kg s.c. (Fig. 2A). The ability of naloxone to precipitate withdrawal (e.g., wet-dog shakes) indicated that the morphine-pelleted mice were physically dependent (Fig. 2B). However, the morphine-pelleted animals retained full analgesic sensitivity to IBNtxA (Fig. 2C), as shown by the absence of a shift of the dose-response curve. Furthermore, administration of IBNtxA to the morphine-pelleted animals failed to demonstrate any evidence of withdrawal (e.g., wet-dog shakes).

Respiratory depression remains one of the most problematic opioid side effects. Although low morphine doses are used to ameliorate dyspnea in a range of pulmonary problems, higher doses of opioids can be life threatening. In contrast to morphine, IBNtxA does not affect the respiratory rate in mice (Majumdar et al., 2011b). We now examined its effects in rats, using oximetry to determine oxygen saturation levels. Using doses of drug approximately 4- to 5-fold greater than their respective analgesic ED₅₀, we compared IBNtxA to morphine (Fig. 3). As anticipated, the high morphine dose significantly depressed oxygen saturation, lowering it by 30%, a symptomatic clinical value. In marked contrast, an equianalgesic IBNtxA dose was indistinguishable from the saline group, displaying no evidence of any respiratory depression.

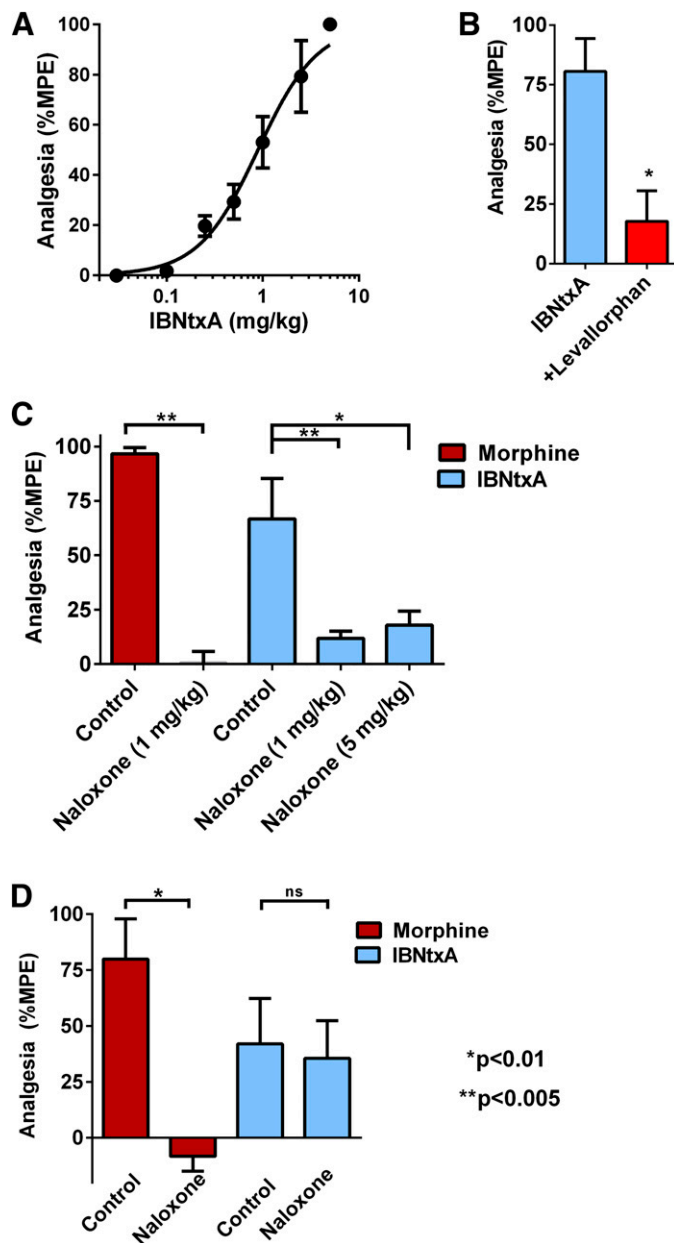


Fig. 1. IBNtxA analgesia and sensitivity toward naloxone. (A) Groups of rats ($n = 4$) were assessed for IBNtxA analgesia at peak effect in three independent experiments ($n = 12$ total) in a cumulative dose-response paradigm where animals received escalating doses of IBNtxA to generate the analgesic dose-response curve. The ED₅₀ was 0.89 mg/kg (95% confidence interval, 0.69–1.2). (B) Groups of rats ($n = 8$) received IBNtxA (2 mg/kg) and were tested for analgesia after 30 minutes, immediately given levallorphan (1 mg/kg s.c.), and tested 20 minutes later. (C) Groups of rats ($n = 4$) received either morphine (8 mg/kg s.c.) or IBNtxA (2 mg/kg s.c.) immediately after the injection of saline or the indicated dose of naloxone. Naloxone significantly reversed morphine analgesia (Bonferroni multiple comparison test, $P < 0.0001$) as well as IBNtxA ($P < 0.005$ at 1 mg/kg, $P < 0.01$ at 5 mg/kg). (D) To assess the effect of a low naloxone dose, groups of rats were injected with equivalent analgesic doses of morphine 8 mg/kg s.c. ($n = 4$) or IBNtxA 2 mg/kg s.c. ($n = 5$). Animals were tested in the tail-flick assay after 20 minutes for morphine and 30 minutes for IBNtxA. They were then immediately administered naloxone (0.1 mg/kg s.c.) and tested again in the tail-flick assay 20 minutes later. Results are reported as percentage of maximum possible effect (%MPE). The results for morphine revealed a significant reduction by naloxone (paired t test, $P < 0.005$). There was no significant (ns) naloxone effect against IBNtxA.

IBNtxA Binding Sites. In the mouse, [¹²⁵I]IBNtxA labels a target in the brain with high affinity that is pharmacologically

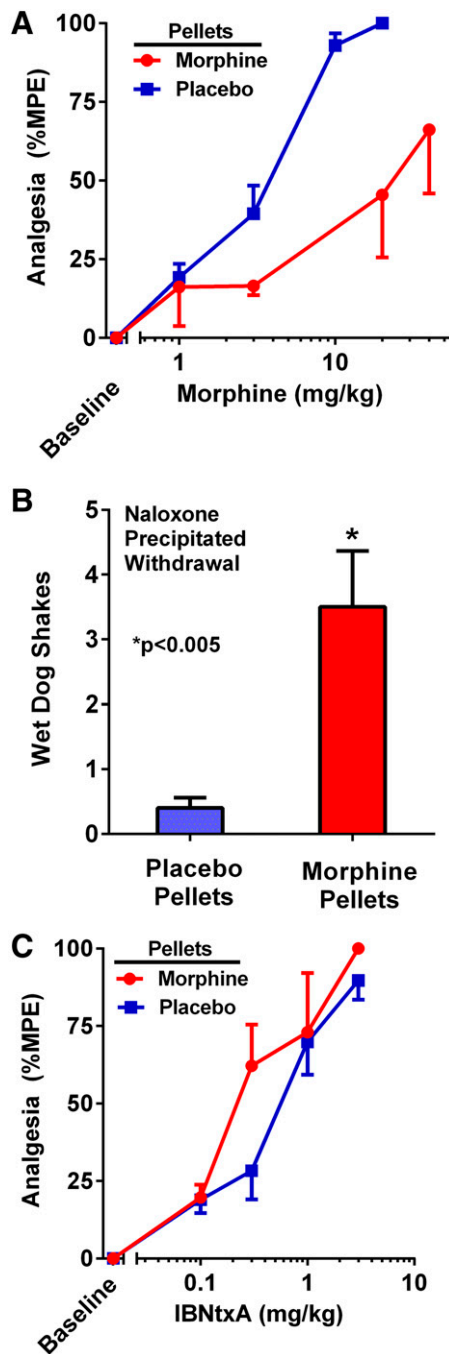


Fig. 2. Assessment of cross-tolerance between morphine and IBNtxA. Analgesic cross-tolerance was assessed in groups of rats, each implanted with either three morphine ($n = 4$) or placebo ($n = 10$) pellets and tested with IBNtxA 72 hours later. (A) The next day cumulative morphine dose-response curves revealed a morphine ED_{50} of 20.5 mg/kg (95% confidence interval [CI], 7.7–54) in the morphine-pelleted rats compared with 3.3 mg/kg (95% CI, 2.7–4.0) in placebo-pelleted animals ($P < 0.001$). (B) After the morphine cumulative dose-response testing, animals received naloxone (1 mg/kg s.c.). Morphine-pelleted animals displayed a significantly greater number of wet-dog shakes relative to placebo-pelleted controls ($P < 0.005$). (C) In morphine-pelleted animals, the ED_{50} for IBNtxA was 0.26 mg/kg (95% CI, 0.15–0.45) compared with 0.52 mg/kg (95% CI, 0.37–0.75) in the placebo-pelleted rats. %MPE, percentage of maximum possible effect.

distinct from any of the traditional opioid receptors (Majumdar et al., 2011b). Studies in a series of KO mice indicate that the binding site is dependent on a set of μ -opioid receptor splice

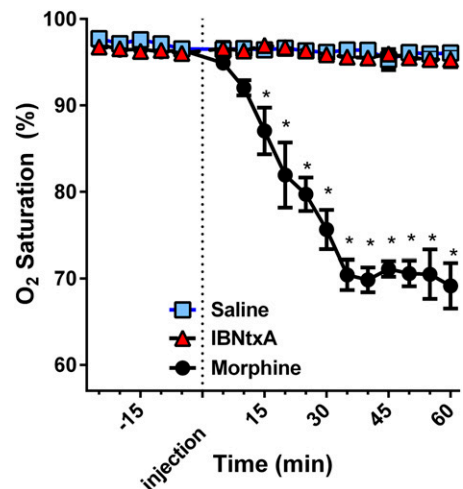


Fig. 3. Effect of IBNtxA on respiratory function. Rats were randomly assigned to receive saline ($n = 4$), IBNtxA (4 mg/kg, $n = 4$), or morphine (20 mg/kg, $n = 4$). Using oximetry, each animal's O_2 saturation was measured for 5 seconds every 5 minutes for 25 minutes before and 60 minutes after injection of the treatment. There were no differences between IBNtxA and saline at any time point, whereas morphine significantly depressed O_2 saturation (* $P < 0.0001$ at each time point) from 15–60 minutes as determined by Bonferroni multiple-comparison test after a repeated-measures analysis of variance.

variants associated with exon 11 that contain only six transmembrane domains. The binding site is insensitive to selective μ , δ , and κ_1 opioids and persists in a triple KO mouse lacking δ (DOR-1), κ_1 (KOR-1), and traditional full-length MOR-1 variants but which still expresses exon 11-associated truncated ones. [125 I]IBNtxA binding in the rat brain displayed a unique binding site with similarities to the one in mice.

Because knockouts of the traditional μ , δ , and κ_1 sites are not available with rats, we employed a blocking strategy to examine these sites. In the mouse, the selective agents CTAP (μ), DPDPE (δ), and U50,488H (κ_1) showed minimal activity against [125 I]IBNtxA binding in the triple KO animals (Majumdar et al., 2011b). We therefore examined [125 I]IBNtxA binding in the presence of high concentrations of the three selective compounds (250 nM each). In the presence of these blocking concentrations of CTAP, DPDPE, and U50,488H, [125 I]IBNtxA still labeled with high affinity a binding site in rat brain homogenates ($K_D = 0.22 \pm 0.02$ nM) (Fig. 4A). Although the observed affinity for the site is virtually identical to that seen in mouse triple KO brain ($K_D = 0.16 \pm 0.04$ nM), the B_{max} in rats (15 ± 2 fmol/mg protein) was approximately 4-fold lower than in the triple KO mice ($B_{max} = 60.8 \pm 1.6$ fmol/mg protein) (Majumdar et al., 2011b), perhaps explaining the lower analgesic potency in the rat relative to the mouse.

The binding selectivity profile of the [125 I]IBNtxA site differed from traditional opioid receptors and showed similarities to those seen in the mouse (Table 1). Highly selective μ , δ , and κ_1 drugs displayed very poor affinity for the site. Yet a variety of other opioids that had previously been associated with the κ_3 site (Clark et al., 1989) displayed far greater affinity. Many of these drugs displayed shallow competition curves, as determined by logit plots (Hill slopes) suggesting that [125 I]IBNtxA might be labeling more than one type of site. Selected compounds were chosen for detailed competition curves (Fig. 5; Table 2). The competition curves of the four

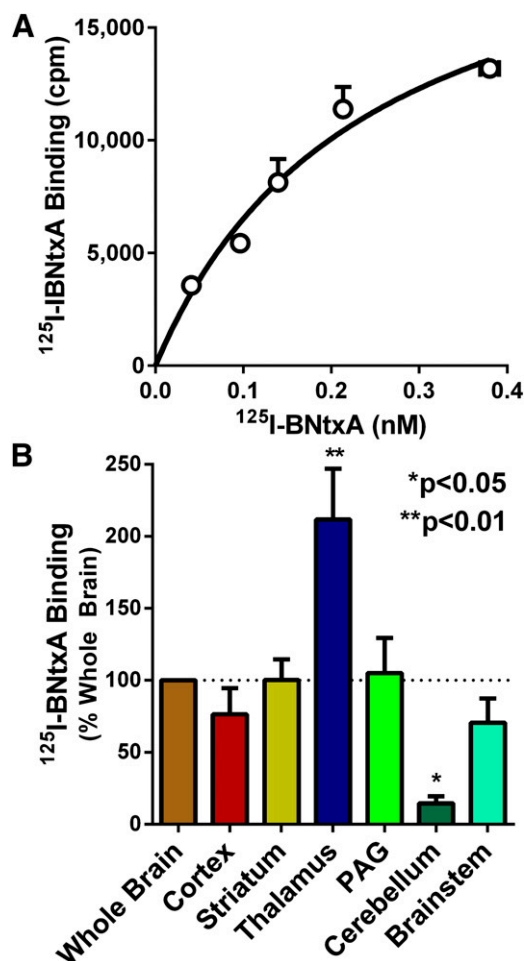


Fig. 4. [^{125}I]IBNtxA binding in rat brain. (A) Saturation studies were performed using brain homogenates in the presence of μ (CTAP), δ (DPDPE), or κ_1 (U50,488H) blockers at 250 nM. Nonspecific binding was defined by levallorphan (1 μM). Only specific binding is reported. The assay was replicated 3 times, and the results shown are from a representative experiment. Nonlinear regression analysis using GraphPad Prism generated an average K_D from the three independent experiments of 0.22 ± 0.02 nM and a B_{max} of 15 ± 2 fmol/mg protein. (B) Rat brains were rapidly removed and dissected into the indicated regions in ice-cold phosphate-buffered saline, and homogenates were prepared. Binding in each region was determined using [^{125}I]IBNtxA (0.1 nM) in the presence of μ , δ , and κ_1 blockers (250 nM each) and normalized to binding from the whole brain. Results are the mean of three independent experiments. Binding differed across regions (one-way analysis of variance, $P = 0.0003$), with the thalamus showing significantly more binding than the whole-brain control (Bonferroni multiple comparison test, $P < 0.01$) and the cerebellum showing significantly less binding than the whole-brain control ($P < 0.05$). PAG, periaqueductal gray.

compounds were clearly biphasic, with a high-affinity and a low-affinity component (Fig. 5). When submitted to nonlinear regression analysis, all four compounds were best fit to a two-site model, with the high-affinity component corresponding to approximately a third of the overall B_{max} . The affinity of levallorphan for the mouse site corresponded closely to the high-affinity component in the rat. The high-affinity binding component of the other compounds in the rat displayed affinities at least as great as in the mouse. Of particular interest are naltrindole and norbinaltorphimine (norBNI). Although these drugs are considered highly selective δ and κ_1 antagonists, respectively, they also competed a component of [^{125}I]IBNtxA site with subnanomolar affinity.

Finally, we examined the regional distribution of these binding sites. After the dissection of various regions, we performed homogenate binding studies (Fig. 4B). Binding was readily demonstrated in all the regions examined. The levels in cortex, striatum, periaqueductal gray, and brainstem were quite similar to those in whole brain. However, the binding in the thalamus was over 2-fold greater ($P < 0.01$) and in the cerebellum was 85% lower ($P < 0.05$).

Discussion

Much effort has been dedicated to the development of opioids that lack side effects and potential for abuse. The ability to dissociate morphine analgesia from many of its side effects, including respiratory depression, constipation, and physical dependence, were documented almost 30 years ago (Ling et al., 1983, 1984, 1985, 1989; Heyman et al., 1988; Paul and Pasternak, 1988). However, only recently have inroads been made in the synthesis of selective compounds. Two general approaches have been used. One involves the role of biased agonism, with biased drugs showing differences in pharmacologic actions based upon their relative activation of β -arrestin compared with G protein mechanisms (Bohn et al., 2002, 2003; Violin and Lefkowitz, 2007; Reiter et al., 2012; Dewire et al., 2013). The ability to differentiate opioid actions mediated through the same binding site with biased signaling offers major advantages in the design of ligands for G protein-coupled receptors.

The demonstration of heterodimerization of opioid receptors has yielded several interesting approaches toward the development of novel opioids. Opioid heterodimers can form targets with unique binding and pharmacologic profiles. MOR-1/DOR-1 dimers have been proposed (Gomes et al., 2013; Ferre et al., 2014), as has a DOR-1/KOR-1 dimer (Jordan and Devi, 1999) that corresponds to the pharmacologically defined κ_2 receptor (Zukin et al., 1988). MOR-1 also dimerizes with opioid receptor-like 1 receptors to generate a target with a novel pharmacologic profile (Pan et al., 2002). More recently, an intriguing compound was reported that targets a MOR-1/KOR-1 heterodimer (Yekkirala et al., 2011). *N*-Naphthoyl- β -naltrexamine is a potent analgesic that lacks physical dependence or reward behavior. Structurally, it is similar to IBNtxA in that it, too, is based upon the 6 β -naltrexamine scaffold. However, they differ in their mechanisms of action. *N*-Naphthoyl- β -naltrexamine acts through a heterodimer of MOR-1 and KOR-1 while IBNtxA retains full analgesic activity in triple KO mice that lack KOR-1 (Yekkirala et al., 2011).

The extensive alternative splicing of the μ -opioid receptor gene with its three classes of variants offers additional targets for drug development (for a review, see Pasternak and Pan, 2013). The full-length variants with their alternative 3'-splicing all bind traditional μ opioids. Their different C terminus sequences influence their distribution within the cell (Abbadie et al., 2001) and among regions (Abbadie et al., 2000a,b) and their intrinsic activity (Bolan et al., 2004) and even their ability to internalize (Caron et al., 2000; Abbadie and Pasternak, 2001; Tanowitz et al., 2008). Furthermore, each variant is also subject to biased signaling, further extending their possibilities.

The truncated exon 11-associated 6TM variant class that we initially described over a decade ago (Pan et al., 2001) offers another approach toward the development of improved

TABLE 1

Competition of [¹²⁵I]IBNtxA binding in rat brain by a series of opioids

Rat brain homogenates were prepared and binding assays performed in the presence of μ , δ , and κ_1 blockers, as described in *Materials and Methods*. Results are the mean \pm S.E.M. of three independent determinations, each performed in triplicate. Hill slopes were determined on the binding between 15 and 85% inhibition. A number of compounds showed slopes far less than 1, consistent with binding heterogeneity, indicating that their K_i values may reflect the cumulative inhibition of different affinity sites. Several were examined in further detail (Table 2). The values for the triple KO mouse brain (Majumdar et al., 2011b), calf brain (Clark et al., 1989), and human neuroblastoma SK-N-BE(2)-C cell line (Standifer et al., 1994) are taken from the literature.

Drug	[¹²⁵ I]IBNtxA		[³ H]NalBzoH (κ_3)		
	Rat		Triple KO Mouse Brain	Calf Striatum	Human BE(2)-C Cells
	K_i	Hill Slope	K_i	K_i	K_i
	<i>nM</i>		<i>nM</i>	<i>nM</i>	<i>nM</i>
μ					
Morphine	530 \pm 130	-0.70 \pm 0.18	>1000	32.8 \pm 2.2	11.1 \pm 4
Fentanyl	201 \pm 92	-0.72 \pm 0.23	226 \pm 40		
DAMGO	>1000		>1000	8.2 \pm 1.9	18.3 \pm 4.5
CTAP	>1000		>1000		
Naloxone	373 \pm 156	-0.83 \pm 0.19	51.9 \pm 1.4	8.4 \pm 0.9	13.8 \pm 6
δ					
Naltrindole	43.3 \pm 11	-0.50 ^a	26.3 \pm 2.3		25.6 \pm 8.7
DPDPE	>1000		>1000	>350	>1000
κ_1					
U50,488	>1000		>1000	>350	>1000
norBNI	651 \pm 350	-0.53 \pm 0.25	3.3 \pm 1.9	103 \pm 85	71 \pm 9.6
Other opioid					
Levorphanol	90 \pm 42	-0.55 \pm 0.32	8.8 \pm 2.5		
Ketocyclazocine	150 \pm 34	-0.83 \pm 0.18	0.04 \pm 0.01	4.5 \pm 0.8	
Ethylketocyclazocine	5.0 \pm 1.5	-0.74 \pm 0.06	0.21 \pm 0.11	1.4 \pm 0.5	
Nalorphine	52 \pm 19	-0.63 \pm 0.44	3.71 \pm 1.45		
NalBzoH	30 \pm 7	-0.75 \pm 0.16	0.59 \pm 0.15	0.9 \pm 0.19	
Levallorphan	23 \pm 13	-0.49 \pm 0.28	0.34 \pm 0.02	2.2 \pm 0.56	
Buprenorphine	5.7 \pm 0.7	-0.65 \pm 0.24	1.8 \pm 0.93		
σ_1					
(+)-Pentazocine	>1000		>1000		

DAMGO, Tyr-D-Ala-Gly-N-methyl-Phe-Gly-ol.

^aThis value represents a single determination.

analgesics. Using a scaffold based upon either naltrexone (Majumdar et al., 2011a,b) or naloxone (Majumdar et al., 2012), we generated a series of compounds capable of acting through the 6TM variants of mice with very unique pharmacologic profiles, including IBNtxA. In mice, IBNtxA is a potent analgesic lacking many of the side effects of traditional opioids. To investigate whether this advantageous pharmacologic profile seen in mice extends to other species also expressing six transmembrane domain receptors, we examined the pharmacology of IBNtxA in rats.

Our results establish a similar pharmacology between rats and mice, although rats are somewhat less sensitive, which may be related to their lower density of [¹²⁵I]IBNtxA-binding sites relative to mice. IBNtxA is a potent analgesic in rats. At doses as high as 5-fold its analgesic ED₅₀, it fails to show any changes in oxygen saturation, a sensitive measure of respiratory depression, and it shows no cross-tolerance to morphine. Furthermore, when administered to highly morphine-dependent animals, it did not precipitate any withdrawal signs (Majumdar et al., 2011a).

The target responsible for the actions of IBNtxA remains unclear. In both mice and rats, [¹²⁵I]IBNtxA labels a non-traditional site insensitive to traditional selective μ (morphine and Tyr-D-Ala-Gly-N-methyl-Phe-Gly-ol), δ (DPDPE), or κ_1 (U50,488H) sites. The studies in mice, which have the advantage of a range of genetic models, strongly indicate a role for truncated 6TM splice variants of MOR-1 in the non-traditional [¹²⁵I]IBNtxA binding sites, which persist in the triple KO mice and are lost in the exon 11 KO mouse. Like mice and humans, rats also express truncated 6TM variants.

However, the relative abundance of these binding sites in the rat brain (15 fmol/mg protein) is only one-fourth that seen in the triple KO mice (60.8 fmol/mg protein) and far lower than levels seen in BE(2)-C human neuroblastoma cell lines (30–75 fmol/mg protein). The lower expression levels in the rat may help explain the lower analgesic potency of the drug.

Comparing the binding-selectivity profile in the mouse and rat reveals important similarities and some differences. In both species, the non-traditional [¹²⁵I]IBNtxA binding site is not competed by many classic μ -, δ -, and κ_1 -selective agents. [¹²⁵I]IBNtxA binding in the rat is heterogeneous, based upon the shallow competition curves and the Hill coefficients less than unity. The full curves appear biphasic, and curve fitting supports two sites. When dissected into two components by nonlinear regression analysis, approximately a third of [¹²⁵I]IBNtxA binding is quite sensitive to naltrindole, levallorphan, norBNI, and levorphanol, with K_i values well below 1 nM, with the remainder of the binding relatively insensitive. The presence of more than one population of labeled sites in the rat illustrates how an overall IC₅₀ that is used to generate an “apparent” K_i value can be misleading. The overall lower levels of [¹²⁵I]IBNtxA binding in the rat may also contribute to the differences between the values in the mouse and rat, particularly if they change the relative abundance of the two sites. Thus, the estimations of the “ K_i ” values generated from total [¹²⁵I]IBNtxA binding must be interpreted cautiously.

Naltrindole and norBNI are considered selective antagonists for δ and κ receptors, respectively. [¹²⁵I]IBNtxA binding in both mice and rats is modestly sensitive to naltrindole.

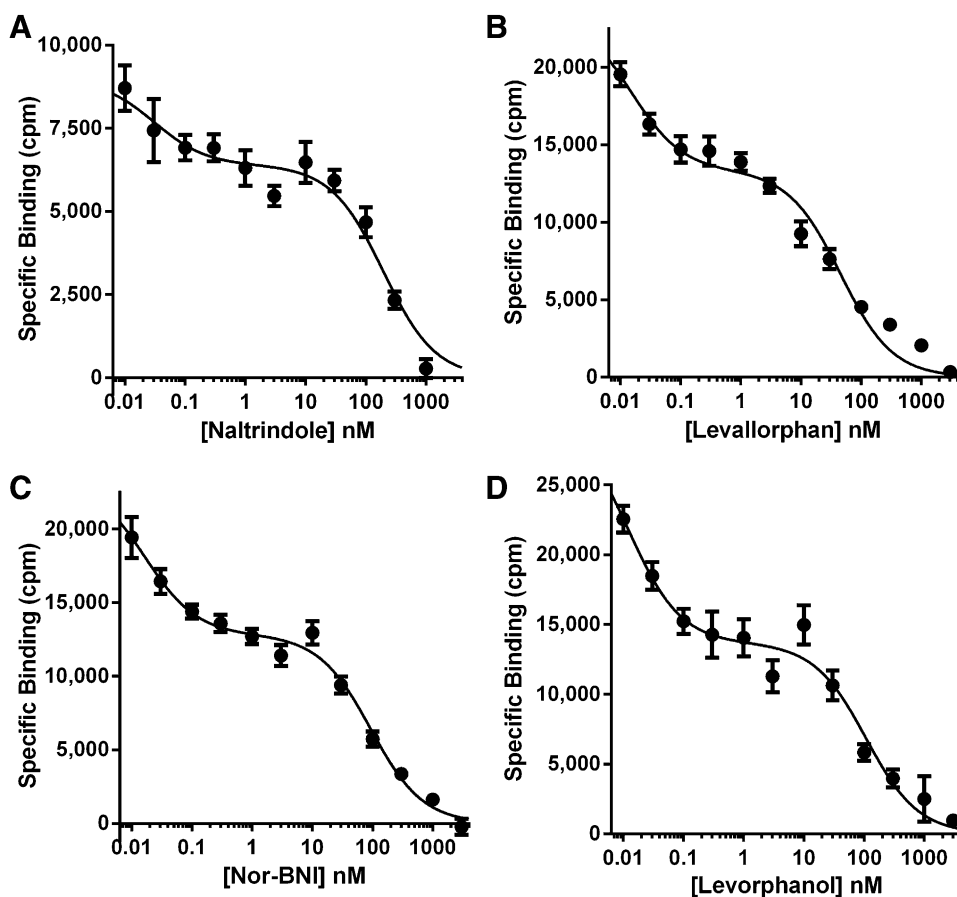


Fig. 5. [125 I]IBNtxA competition studies. Rat brain homogenate was incubated with [125 I]IBNtxA (0.1 nM) with blockers at the indicated concentrations of the specified competitor. Nonspecific binding was determined using levallorphan (1 μ M). Results are from three independent replications. Each data set was fit with both a one-site and two-site model using GraphPad Prism, and the models were compared using an extra-sum-of-squares *F*-test. The two-site model was preferred for each of the four drugs tested ($P < 0.0001$ for each).

However, approximately a third of the binding in the rat is competed by naltrindole with a K_i of 60 pM. It seems unlikely that this reflects labeling of δ receptors, considering the inclusion of 250 nM DPDPE. A similar situation is revealed by norBNI competing a portion of [125 I]IBNtxA binding very potently (K_i 80 pM). Again, the presence of 250 nM U50,488H argues against labeling traditional κ_1 receptors, raising questions on the selectivity of both compounds.

The rat expresses two 6TM variants, rMOR-1G1 and rMOR-1G2. Both contain exon 4 but differ due to 5'-splicing. rMOR-1G2 yields a typical 6TM variant containing the 5' coding sequence from exon 11. However, rMOR-1G1 splicing is more complicated because the AUG start codon within exon 11 yields a very short peptide of only seven amino acids due to a frame shift. It is still possible to generate a 6TM using the AUG start codon at the beginning of exon 2, in a manner similar to that previously reported for the μ_3 receptor (Cadet et al., 2003; Pasternak and Pan, 2013). Although the diversity

of binding might be due to these two variants, this seems unlikely because they only differ at the N terminus by the presence of seven amino acids encoded by exon 11 in rMOR-1G2 (MGSGPML) that would be absent in rMOR-1G1. An alternative explanation may involve heterodimerization of a 6TM variant with more than one nonopioid G protein-coupled receptor, leading to the possibility of a family of 6TM-related complexes.

In many respects, the [125 I]IBNtxA target resembles the previously reported κ_3 sites (Clark et al., 1989). Drugs earlier defined pharmacologically as κ_3 , including levorphanol (Moulin et al., 1988; Tive et al., 1992), nalorphine (Paul et al., 1991), and NalBzoH (naloxone benzoylhydrazone) (Luke et al., 1988; Gistrak et al., 1989; Paul et al., 1990), compete [125 I]IBNtxA binding in the triple KO mouse quite potently (Majumdar et al., 2011b). Equally important, their analgesic actions are greatly diminished in the exon 11 KO mouse, as is IBNtxA (Majumdar et al., 2011b). Thus, NalBzoH analgesia is dependent upon

TABLE 2

Detailed competition studies of selected opioids on [125 I]IBNtxA binding

Rat brain homogenates were prepared and binding assays performed as described in *Materials and Methods*. Results are the mean \pm S.E.M. of three independent determinations, each performed in triplicate. Competitions were best fit by nonlinear regression analysis.

Drug	K_i High Affinity	K_i Low Affinity	Fraction of High-Affinity Sites	Two-Site versus One-Site Model <i>P</i> Value
	<i>nM</i>			
Naltrindole	0.06 \pm 0.03	321 \pm 160	27 \pm 1%	<0.001
Levallorphan	0.12 \pm 0.03	119 \pm 26	35 \pm 5%	<0.001
norBNI	0.08 \pm 0.01	184 \pm 23	34 \pm 7%	<0.001
Levorphanol	0.07 \pm 0.01	219 \pm 44	42 \pm 8%	<0.001

6TM variants. However, there are some differences between the κ_3 -binding sites defined with [^3H]NaIBzoH and the [^{125}I]IBNtxA target, both of which are also seen in human BE(2)-C neuroblastoma cells (Clark et al., 1989; Standifer et al., 1994; Mathis et al., 2001).

The regional expression of [^{125}I]IBNtxA binding varies. Homogenate binding studies show high levels in the thalamus and low levels in the cerebellum. The resolution of the anatomic distributions in the current autoradiography studies is relatively limited, but the pattern in the rat is similar to that in the triple KO mouse seen autoradiographically, which expresses none of the traditional opioid receptors (S. Grinnell, S. Majumdar, V. Le Rouzic, M. Ansonoff, J. E. Pintar, and G. W. Pasternak, submitted manuscript). Thus, the labeling in the rat is consistent with a target including 6TM variants.

In summary, [^{125}I]IBNtxA has identified a unique target in the rat similar to the target in the triple KO mouse. Working through this target, IBNtxA produces a potent analgesia in the radiant heat tail-flick assay in both the rat and mouse. Additional studies in the mouse indicate that IBNtxA also is potent in inflammatory and neuropathic hypersensitivity and spontaneous pain (Wieskopf et al., in press). Unlike traditional opioids, IBNtxA produces its analgesia in the rat without producing respiratory depression or physical dependence. It shows no cross-tolerance to morphine and does not precipitate withdrawal in morphine-dependent animals. Together, these observations in both the rat and the mouse suggest that the truncated 6TM variants may provide a valuable target for the development of novel analgesics lacking the limitations of current drugs.

Authorship Contributions

Participated in research design: Grinnell, Majumdar, Pasternak.

Conducted experiments: Grinnell, Majumdar, Narayan, Le Rouzic, Ansonoff.

Contributed new reagents or analytic tools: Majumdar, Ansonoff, Pintar, Pasternak.

Performed data analysis: Grinnell, Narayan, Le Rouzic, Pasternak.

Wrote or contributed to the writing of the manuscript: Grinnell, Pasternak.

References

- Abbadie C, Pan Y, Drake CT, and Pasternak GW (2000a) Comparative immunohistochemical distributions of carboxy terminus epitopes from the mu-opioid receptor splice variants MOR-1D, MOR-1 and MOR-1C in the mouse and rat CNS. *Neuroscience* **100**:141–153.
- Abbadie C, Pan Y, and Pasternak GW (2000b) Differential distribution in rat brain of mu opioid receptor carboxy terminal splice variants MOR-1C-like and MOR-1-like immunoreactivity: evidence for region-specific processing. *J Comp Neurol* **419**:244–256.
- Abbadie C and Pasternak GW (2001) Differential in vivo internalization of MOR-1 and MOR-1C by morphine. *Neuroreport* **12**:3069–3072.
- Abbadie C, Pasternak GW, and Aicher SA (2001) Presynaptic localization of the carboxy-terminus epitopes of the mu opioid receptor splice variants MOR-1C and MOR-1D in the superficial laminae of the rat spinal cord. *Neuroscience* **106**:833–842.
- Bare LA, Mansson E, and Yang D (1994) Expression of two variants of the human μ opioid receptor mRNA in SK-N-SH cells and human brain. *FEBS Lett* **354**:213–216.
- Bohn LM, Gainetdinov RR, Sotnikova TD, Medvedev IO, Lefkowitz RJ, Dykstra LA, and Caron MG (2003) Enhanced rewarding properties of morphine, but not cocaine, in beta(arrestin)-2 knock-out mice. *J Neurosci* **23**:10265–10273.
- Bohn LM, Lefkowitz RJ, and Caron MG (2002) Differential mechanisms of morphine antinociceptive tolerance revealed in (beta)arrestin-2 knock-out mice. *J Neurosci* **22**:10494–10500.
- Bolan EA, Pan YX, and Pasternak GW (2004) Functional analysis of MOR-1 splice variants of the mouse mu opioid receptor gene *Oprm*. *Synapse* **51**:11–18.
- Cadet P, Mantione KJ, and Stefano GB (2003) Molecular identification and functional expression of mu 3, a novel alternatively spliced variant of the human mu opiate receptor gene. *J Immunol* **170**:5118–5123.
- Caron MG, Bohn LM, Barak LS, Abbadie C, Pan Y-X and Pasternak GW (2000) Mu opioid receptor spliced variant, MOR1D, internalizes after morphine treatment in HEK-293 cells in *Abstracts: Society for Neuroscience 30th Annual Meeting*; 2000 Nov 4–9; New Orleans, LA. no. 783.3, Society for Neuroscience, Washington, DC.
- Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099–3108.
- Cicero TJ and Meyer ER (1973) Morphine pellet implantation in rats: quantitative assessment of tolerance and dependence. *J Pharmacol Exp Ther* **184**:404–408.
- Clark JA, Houghten R, and Pasternak GW (1988) Opiate binding in calf thalamic membranes: a selective mu 1 binding assay. *Mol Pharmacol* **34**:308–317.
- Clark JA, Liu L, Price M, Hersh B, Edelson M, and Pasternak GW (1989) Kappa opiate receptor multiplicity: evidence for two U50,488-sensitive kappa1 subtypes and a novel kappa3 subtype. *J Pharmacol Exp Ther* **251**:461–468.
- DeWire SM, Yamashita DS, Rominger DH, Liu G, Cowan CL, Graczyk TM, Chen XT, Pitis PM, Gotchev D, and Yuan C, et al. (2013) A G protein-biased ligand at the μ -opioid receptor is potentially analgesic with reduced gastrointestinal and respiratory dysfunction compared with morphine. *J Pharmacol Exp Ther* **344**:708–717.
- Du Y-L, Elliot K, Pan Y-X, Pasternak GW, and Inturrisi CE (1997) A splice variant of the mu opioid receptor is present in human SHSY-5Y cells in *Abstracts: Society for Neuroscience 27th Annual Meeting*; 1997 Oct 25–29; New Orleans, LA. p 1206, Society for Neuroscience, Washington, DC.
- Ferré S, Casadó V, Devi LA, Filizola M, Jockers R, Lohse MJ, Milligan G, Pin JP, and Guitart X (2014) G protein-coupled receptor oligomerization revisited: functional and pharmacological perspectives. *Pharmacol Rev* **66**:413–434.
- Gistrak MA, Paul D, Hahn EF, and Pasternak GW (1989) Pharmacological actions of a novel mixed opiate agonist/antagonist: naloxone benzoylhydrazone. *J Pharmacol Exp Ther* **251**:469–476.
- Gomes I, Fujita W, Gupta A, Saldanha SA, Negri A, Pinello CE, Eberhart C, Roberts E, Filizola M, and Hodder P, et al. (2013) Identification of a μ - δ opioid receptor heteromer-biased agonist with antinociceptive activity. *Proc Natl Acad Sci USA* **110**:12072–12077.
- Hahn EF, Carroll-Buatti M, and Pasternak GW (1982) Irreversible opiate agonists and antagonists: the 14-hydroxydihydromorphinone azines. *J Neurosci* **2**:572–576.
- Hahn EF and Pasternak GW (1982) Naloxonazine, a potent, long-lasting inhibitor of opiate binding sites. *Life Sci* **31**:1385–1388.
- Heyman JS, Williams CL, Burks TF, Mosberg HI, and Porreca F (1988) Dissociation of opioid antinociception and central gastrointestinal propulsion in the mouse: studies with naloxonazine. *J Pharmacol Exp Ther* **245**:238–243.
- Jordan BA and Devi LA (1999) G-protein-coupled receptor heterodimerization modulates receptor function. *Nature* **399**:697–700.
- Ling GSF, MacLeod JM, Lee S, Lockhart SH, and Pasternak GW (1984) Separation of morphine analgesia from physical dependence. *Science* **226**:462–464.
- Ling GSF, Paul D, Simantov R, and Pasternak GW (1989) Differential development of acute tolerance to analgesia, respiratory depression, gastrointestinal transit and hormone release in a morphine infusion model. *Life Sci* **45**:1627–1636.
- Ling GSF, Simantov R, Clark JA, and Pasternak GW (1986) Naloxonazine actions in vivo. *Eur J Pharmacol* **129**:33–38.
- Ling GSF, Spiegel K, Lockhart SH, and Pasternak GW (1985) Separation of opioid analgesia from respiratory depression: evidence for different receptor mechanisms. *J Pharmacol Exp Ther* **232**:149–155.
- Ling GSF, Spiegel K, Nishimura SL, and Pasternak GW (1983) Dissociation of morphine's analgesic and respiratory depressant actions. *Eur J Pharmacol* **86**:487–488.
- Litchfield JT, Jr and Wilcoxon F (1949) A simplified method of evaluating dose-effect experiments. *J Pharmacol Exp Ther* **96**:99–113.
- Luke MC, Hahn EF, Price M, and Pasternak GW (1988) Irreversible opiate agonists and antagonists: V. Hydrazone and acylhydrazone derivatives of naltrexone. *Life Sci* **43**:1249–1256.
- Majumdar S, Burgman M, Haselton N, Grinnell S, Ocampo J, Pasternak AR, and Pasternak GW (2011a) Generation of novel radiolabeled opiates through site-selective iodination. *Bioorg Med Chem Lett* **21**:4001–4004.
- Majumdar S, Grinnell S, Le Rouzic V, Burgman M, Polikar L, Ansonoff M, Pintar J, Pan YX, and Pasternak GW (2011b) Truncated G protein-coupled mu opioid receptor MOR-1 splice variants are targets for highly potent opioid analgesics lacking side effects. *Proc Natl Acad Sci USA* **108**:19778–19783.
- Majumdar S, Subrath J, Le Rouzic V, Polikar L, Burgman M, Nagakura K, Ocampo J, Haselton N, Pasternak AR, and Grinnell S, et al. (2012) Synthesis and evaluation of aryl-naloxamide opiate analgesics targeting truncated exon 11-associated μ opioid receptor (MOR-1) splice variants. *J Med Chem* **55**:6352–6362.
- Mathis JP, Mandym CD, Altememi GF, Pasternak GW, and Standifer KM (2001) Orphanin FQ/nociceptin and naloxone benzoylhydrazone activate distinct receptors in BE(2)-C human neuroblastoma cells. *Neurosci Lett* **299**:173–176.
- Moulin DE, Ling GSF, and Pasternak GW (1988) Unidirectional analgesic cross-tolerance between morphine and levorphanol in the rat. *Pain* **33**:233–239.
- Pan YX, Bolan E, and Pasternak GW (2002) Dimerization of morphine and orphanin FQ/nociceptin receptors: generation of a novel opioid receptor subtype. *Biochem Biophys Res Commun* **297**:659–663.
- Pan YX, Xu J, Bolan E, Abbadie C, Chang A, Zuckerman A, Rossi G, and Pasternak GW (1999) Identification and characterization of three new alternatively spliced mu-opioid receptor isoforms. *Mol Pharmacol* **56**:396–403.
- Pan YX, Xu J, Bolan E, Moskowitz HS, Xu M, and Pasternak GW (2005) Identification of four novel exon 5 splice variants of the mouse mu-opioid receptor gene: functional consequences of C-terminal splicing. *Mol Pharmacol* **68**:866–875.
- Pan YX, Xu J, Mahurter L, Bolan E, Xu M, and Pasternak GW (2001) Generation of the mu opioid receptor (MOR-1) protein by three new splice variants of the *Oprm* gene. *Proc Natl Acad Sci USA* **98**:14084–14089.

- Pan YX, Xu J, Xu M, Rossi GC, Matulonis JE, and Pasternak GW (2009) Involvement of exon 11-associated variants of the mu opioid receptor MOR-1 in heroin, but not morphine, actions. *Proc Natl Acad Sci USA* **106**:4917–4922.
- Pasternak DA, Pan L, Xu J, Yu R, Xu MM, Pasternak GW, and Pan YX (2004) Identification of three new alternatively spliced variants of the rat mu opioid receptor gene: dissociation of affinity and efficacy. *J Neurochem* **91**:881–890.
- Pasternak GW, Childers SR, and Snyder SH (1980) Opiate analgesia: evidence for mediation by a subpopulation of opiate receptors. *Science* **208**:514–516.
- Pasternak GW and Pan YX (2013) Mu opioids and their receptors: evolution of a concept. *Pharmacol Rev* **65**:1257–1317.
- Paul D, Levison JA, Howard DH, Pick CG, Hahn EF, and Pasternak GW (1990) Naloxone benzoylhydrazone (NalBzoH) analgesia. *J Pharmacol Exp Ther* **255**:769–774.
- Paul D and Pasternak GW (1988) Differential blockade by naloxonazine of two μ opiate actions: analgesia and inhibition of gastrointestinal transit. *Eur J Pharmacol* **149**:403–404.
- Paul D, Pick CG, Tive LA, and Pasternak GW (1991) Pharmacological characterization of nalorphine, a kappa₃ analgesic. *J Pharmacol Exp Ther* **257**:1–7.
- Reiter E, Ahn S, Shukla AK, and Lefkowitz RJ (2012) Molecular mechanism of β -arrestin-biased agonism at seven-transmembrane receptors. *Annu Rev Pharmacol Toxicol* **52**:179–197.
- Schuller AG, King MA, Zhang J, Bolan E, Pan YX, Morgan DJ, Chang A, Czick ME, Unterwald EM, and Pasternak GW, et al. (1999) Retention of heroin and morphine-6 beta-glucuronide analgesia in a new line of mice lacking exon 1 of MOR-1. *Nat Neurosci* **2**:151–156.
- Standifer KM, Cheng J, Brooks AI, Honrado CP, Su W, Visconti LM, Biedler JL, and Pasternak GW (1994) Biochemical and pharmacological characterization of mu, delta and kappa₃ opioid receptors expressed in BE(2)-C neuroblastoma cells. *J Pharmacol Exp Ther* **270**:1246–1255.
- Tanowitz M, Hislop JN, and von Zastrow M (2008) Alternative splicing determines the post-endocytic sorting fate of G-protein-coupled receptors. *J Biol Chem* **283**:35614–35621.
- Tive L, Ginsberg K, Pick CG, and Pasternak GW (1992) Kappa₃ receptors and levorphanol-induced analgesia. *Neuropharmacology* **31**:851–856.
- Violin JD and Lefkowitz RJ (2007) Beta-arrestin-biased ligands at seven-transmembrane receptors. *Trends Pharmacol Sci* **28**:416–422.
- Wieskopf JS, Pan Y-X, Marcovitz J, Tuttle AH, Majumdar S, Pidakala J, Pasternak GW, and Mogil JS (2014) Broad spectrum analgesic efficacy of IBNtxA is mediated by exon 11-associated splice variants of the mu-opioid receptor gene. *Pain*, in press.
- Wolozin BL and Pasternak GW (1981) Classification of multiple morphine and enkephalin binding sites in the central nervous system. *Proc Natl Acad Sci USA* **78**:6181–6185.
- Xu J, Xu MM, Matulonis J, Rossi G, Pasternak G, and Pan Y-X (2007) Identification and characterization of seven new alternatively spliced variants from the rat mu opioid receptor gene, OPRM in *Abstracts: Society for Neuroscience 37th Annual Meeting*; 2007 Nov 3–7; San Diego, CA. no. 353.10/G35, Society for Neuroscience, Washington, DC.
- Xu J, Xu M, Brown T, Rossi GC, Hurd YL, Inturrisi CE, Pasternak GW, and Pan YX (2013) Stabilization of the μ -opioid receptor by truncated single transmembrane splice variants through a chaperone-like action. *J Biol Chem* **288**:21211–21227.
- Xu J, Xu M, Rossi GC, Pasternak GW, and Pan YX (2011) Identification and characterization of seven new exon 11-associated splice variants of the rat μ opioid receptor gene, OPRM1. *Mol Pain* **7**:9.
- Yekkirala AS, Lunzer MM, McCurdy CR, Powers MD, Kalyuzhny AE, Roerig SC, and Portoghese PS (2011) N-naphthoyl-beta-naltrexamine (NNTA), a highly selective and potent activator of μ /kappa-opioid heteromers. *Proc Natl Acad Sci USA* **108**:5098–5103.
- Yoburn BC, Chen J, Huang T, and Inturrisi CE (1985) Pharmacokinetics and pharmacodynamics of subcutaneous morphine pellets in the rat. *J Pharmacol Exp Ther* **235**:282–286.
- Zimprich A, Bacher B, and Höllt V (1994) Cloning and expression of an isoform of the rmu-opioid receptor (rmuOR1B). *Regul Pept* **54**:347–348 DOI:10.1016/0167-0115(94)90531-2.
- Zimprich A, Simon T, and Höllt V (1995) Cloning and expression of an isoform of the rat μ opioid receptor (rMOR1B) which differs in agonist induced desensitization from rMOR1. *FEBS Lett* **359**:142–146.
- Zukin RS, Eghbali M, Olive D, Unterwald EM, and Tempel A (1988) Characterization and visualization of rat and guinea pig brain kappa opioid receptors: evidence for kappa₁ and kappa₂ opioid receptors. *Proc Natl Acad Sci USA* **85**:4061–4065.

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