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# In vivo and in vitro characterization of naltrindole-derived ligands at the $\kappa$ -opioid receptor

Joseph J Casal-Dominguez<sup>1</sup>, Mary Clark<sup>2</sup>, John R Traynor<sup>2</sup>,  
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## Abstract

Accumulating evidence supports a role for  $\kappa$ -opioid receptor antagonists in the treatment of mood disorders. Standard  $\kappa$ -antagonists have an unusual pharmacodynamic action, with a single injection blocking receptor signaling for several weeks. Here, we have characterized the  $\kappa$ -selective properties of two ligands, 5'-(2-aminomethyl) naltrindole (5'-AMN) and N-((Naltrindol-5-yl) methyl) pentanimidamide (5'-MABN), to identify whether modifications of the naltrindole side chain produces short-acting  $\kappa$ -antagonists. Opioid receptor binding affinity and activity were assessed using [<sup>3</sup>H]-diprenorphine binding, guanosine-5'-O-(3-[<sup>35</sup>S]-thio) triphosphate ([<sup>35</sup>S]-GTPγS) binding and isolated guinea-pig ileum. Pharmacodynamic profiles of 5'-AMN and 5'-MABN (1–10 mg/kg) were investigated using the tail-withdrawal assay and diuresis. Efficacy was also determined in depression- and anxiety-related behavioral paradigms in CD-1 mice. Both 5'-AMN and 5'-MABN had high affinity for  $\kappa$ -receptors ( $K_i$  1.36±0.98 and 0.27±0.08, respectively) and were revealed as potent  $\kappa$ -antagonists ( $pA_2$  7.43 and 8.18, respectively) and  $\mu$ -receptor antagonists ( $pA_2$  7.62 and 7.85, respectively) in the ileum. Contrary to our hypothesis, in vivo, 5'-AMN and 5'-MABN displayed long-lasting antagonist effects in mice, reducing the antinociceptive actions of U50,488 (10 mg/kg) at 28 and 21 days post-injection, respectively. Interestingly, while 5'-AMN and 5'-MABN were not  $\kappa$ -selective, both compounds did show significant antidepressant- and anxiolytic-like effects at 7–14 days post-injection in mice.

## Keywords

Depression, anxiety, kappa opioid receptor, kappa antagonist, mu antagonist, norBNI

## Introduction

$\kappa$ -receptor antagonists have potential as treatments for a range of psychiatric disorders including mood disorders (Berrocoso et al., 2009; Bruchas et al., 2010; Knoll and Carlezon, 2010; Tejeda et al., 2012). The endogenous opioid peptide dynorphin activates  $G_{i/o}$ -coupled  $\kappa$ -receptors and typically decreases neuronal excitability by inhibiting voltage-gated calcium channels and activating voltage-gated potassium channels. Activation of  $\kappa$ -receptors also activates mitogen-activated protein kinase (MAPK) pathways mediated by ERK, JNK and p38 MAPK (Belcheva et al., 1998; Bruchas et al., 2007). The expression of both prodynorphin, the precursor for dynorphin peptides, and  $\kappa$ -receptors is high in brain regions mediating emotional control and stress responses in human and rodent brains (DePaoli et al., 1994; Kitchen et al., 1997; Lin et al., 2006; Mansour et al., 1994).

$\kappa$ -agonists induce dysphoric responses in humans and aversive responses in rodents (Carlezon et al., 2006; Pfeiffer et al., 1986; Todtenkopf et al., 2004).  $\kappa$ -antagonists,  $\kappa$ -receptor gene deletion or prodynorphin gene disruption have antidepressant-like effects in the forced swim test (McLaughlin et al., 2003; Mague et al., 2003; Newton et al., 2002; Shirayama et al., 2004). Acute (e.g. single immobilization stress) or subchronic (e.g. repeated forced swim stress or social defeat stress) stress produces stress-induced immobility in behavioral paradigms that is reduced by treatment with the  $\kappa$ -antagonist norbinaltorphimine (norBNI), and absent in dynorphin<sup>-/-</sup> and  $\kappa$ -receptor<sup>-/-</sup> mice (Bruchas et al., 2007; McLaughlin et al., 2003; McLaughlin et al., 2006a; McLaughlin et al.,

2006b). Thus, endogenous dynorphins are released (Shirayama et al., 2004) and activate  $\kappa$ -receptors during exposure to acute or subchronic stress.

Concern about the feasibility of developing  $\kappa$ -antagonists for therapeutics has centered on an unusual pharmacodynamic property of prototypic  $\kappa$ -selective antagonists. A single injection of norBNI has peak effects occurring ~24 h post-administration, maximal levels maintained for 7–10 days, returning to control levels over 3–4 weeks (Endoh et al., 1992; Jones and Holtzman, 1992). Such a pharmacodynamic profile, limits in vivo behavioral testing and, potentially, clinical trials if the blockade of  $\kappa$ -receptors cannot be readily reversed. A series of naltrindole-based ligands, substituted at the 5'-position with amine and amidine groups, has been synthesized and shown in vitro to have high selectivity for the  $\kappa$ -receptor (Jales et al., 2000; Olmsted et al., 1993; Stevens et al., 2000). Primary amines are known to be readily metabolizable by amine oxidases and to have short-lasting effects, for example

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phenylethylamine (Blaschko, 1952; Sabelli and Javaid, 1995). We hypothesized that such amine derivatives of naltrindole may therefore have a shorter duration of action than standard  $\kappa$ -receptor antagonists. To identify whether modifications of the naltrindole side chain can alter the pharmacodynamics of these  $\kappa$ -antagonists, we performed the first in vivo characterization of 5'-(aminomethyl) naltrindole (5'-AMN) (compound 5, Olmsted et al., 1993) and the closely related amidine N-((Naltrindol-5-yl) methyl) pentanimidamide (5'-MABN) (compound 10b, Stevens et al., 2000) and tested their  $\kappa$ -selectivity in vitro. In addition, their potential as anxiolytics and antidepressants in mouse behavioral paradigms was assessed.

## Methods and materials

### Materials

Cell culture reagents were from Gibco Life Sciences (Grand Island, New York, USA). All other chemicals were analytical grade and purchased from Sigma-Aldrich except: diazepam (Hameln Pharmaceuticals), fluoxetine hydrochloride (Ascent Scientific, UK), guanosine-5'-O-(3-[ $^{35}$ S]-thio) triphosphate ([ $^{35}$ S]-GTP $\gamma$ S) and [ $^3$ H]-diprenorphine (Perkin Elmer, Massachusetts, USA). NorBNI dihydrochloride, 5'-guanidinonaltrindole trifluoroacetic acid (GNTI), 5'-AMN, 5'-MABN were synthesized (Supplementary Material, Figure 1) (>95% purity) in the Department of Pharmacy and Pharmacology, University of Bath.

### Animals

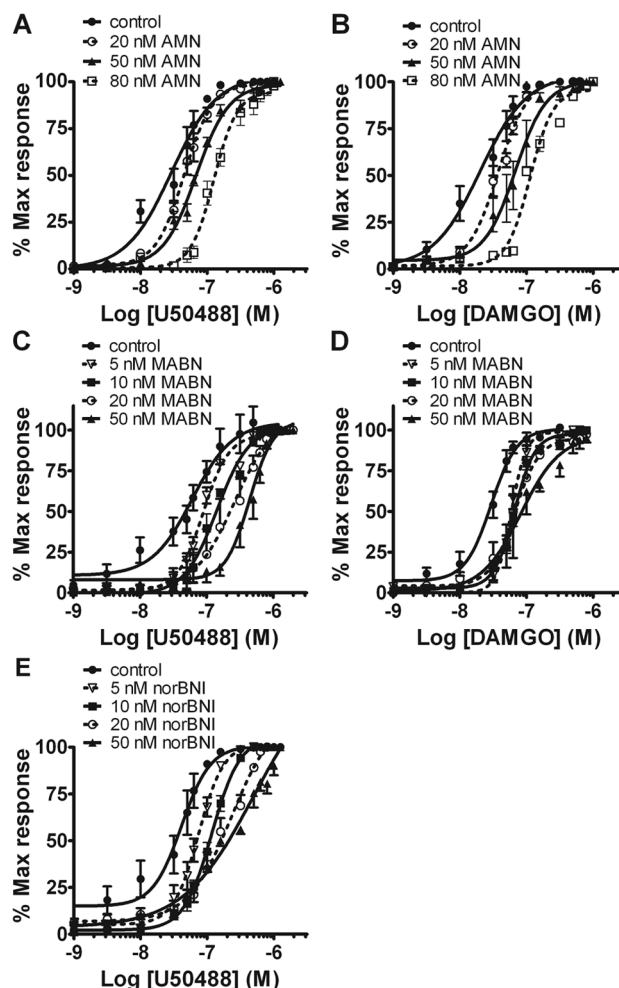
Experiments were performed in accordance with the UK Home Office guidelines and the Animals (Scientific Procedures) Act 1986. Adult (8–9 weeks, 27–38 g) male CD-1 mice, from Charles River (CrI: CD1 (ICR)) and bred at the University of Bath, were housed in groups of 3–4 in cages provided with a shelf, wood shavings and nesting material. Adult (8–9 wks, 250–390 g) male Wistar rats, from Charles River and bred at the University of Bath, were used for diuresis experiments. Rats were housed in groups of four in cages, provided with a tube and wood shavings. The colony rooms were held under a 12 h light/dark cycle (lights on at 07:00) at 20 $\pm$ 2°C with ad libitum food and water. Adult, female, Dunkin Hartley guinea-pigs (300–350 g, Harlan UK) were housed in open floor pens at 19 $\pm$ 2°C, on a 12 h light/dark cycle with ad libitum food and water.

### Cell membrane preparation

Cell membranes were prepared from C<sub>6</sub> rat glioma cells stably transfected with the rat  $\mu$ -receptor (C<sub>6</sub>- $\mu$ ); or  $\delta$ -receptor (C<sub>6</sub>- $\delta$ ); and Chinese hamster ovary (CHO) cells stably expressing the human  $\kappa$ -receptor (CHO- $\kappa$ ) (Clark et al., 1997; Emmerson et al., 1996; Lee et al., 1999).

### [ $^3$ H]-diprenorphine competitive binding assay

Membranes (20  $\mu$ g) were incubated in 50 mM Tris-HCl, pH 7.4 with [ $^3$ H]-diprenorphine (0.2 nM) in the absence or presence of test compounds (norBNI, GNTI, 5'-AMN, and 5'-MABN) with a concentration range of 10<sup>-13</sup>–10<sup>-6</sup> M, for 1 h, in a shaking water bath at 25°C. Nonspecific binding was measured using 50  $\mu$ M



**Figure 1.** Cumulative concentration-response curves, in the isolated guinea-pig ileum for U50,488 and DAMGO, in the presence of increasing concentrations of (A) and (B) 5'-(2-aminomethyl) naltrindole (5'-AMN), (C) and (D) N-((Naltrindol-5-yl) methyl) pentanimidamide (5'-MABN), and (E) norbinaltorphimine (norBNI). All ligands tested caused rightward parallel shifts of agonist concentration-response curves. The results are expressed as the mean percentage of the maximum response induced by the agonist  $\pm$  standard error of the mean (SEM),  $n=4$  tissues.

naloxone. Samples were prepared as described previously (Cami-Kobeci et al., 2009). Data were analyzed using Prism 5.0 (GraphPad Software, California, USA) to determine  $K_i$  values from the IC<sub>50</sub> values using the Cheng-Prusoff equation.

### [ $^{35}$ S]-GTP $\gamma$ S assay

As described previously (Traynor and Nahorski, 1995), C<sub>6</sub>- $\mu$ / $\delta$  or CHO- $\kappa$  membranes (20  $\mu$ g) were incubated in 20 mM Tris-HCl, pH 7.4 buffer containing (mM) 5 MgCl<sub>2</sub>, 100 NaCl, 2.2 dithiothreitol, 30  $\mu$ M GDP, 0.1 nM [ $^{35}$ S]-GTP $\gamma$ S, and 10<sup>-12</sup>–10<sup>-5</sup> M test compound (5'-AMN or 5'-MABN), 10  $\mu$ M U69,593 or 10  $\mu$ M DAMGO or Super Q H<sub>2</sub>O. The membranes were incubated for 60 min in a shaking water bath at 25°C. Samples were prepared as described previously (Cami-Kobeci et al., 2009). [ $^{35}$ S]-GTP $\gamma$ S stimulation of test compounds was expressed as a percentage of

the stimulation by 10  $\mu$ M U69,593 or DAMGO for the  $\kappa$ - and  $\mu$ -receptor, respectively. Antagonist activity ( $K_i$ ) was also determined utilizing a full agonist concentration-response curve of U69,593 or DAMGO in the presence of at least six concentrations of 5'-AMN and 5'-MABN. Data were analyzed using Prism 5.0 (GraphPad Software, California, USA).

### Isolated guinea-pig ileum preparation

Guinea-pigs were killed by CO<sub>2</sub> euthanasia. A piece of ileum was mounted, under 1 g tension to an isotonic transducer, in a 35 mL organ bath at 37°C containing gassed (95% O<sub>2</sub>/5% CO<sub>2</sub>) Krebs solution (mM): 118 NaCl, 11.6 glucose, 25 NaHCO<sub>3</sub>, 4.7 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.2 CaCl<sub>2</sub>·6H<sub>2</sub>O, 1.2 MgSO<sub>4</sub>·7H<sub>2</sub>O. Electrical field stimulation (100 V, 1 ms pulse duration, 0.033 Hz, Grass S-D9 stimulator), was applied for 40 min prior to drug addition. Twitch contractions were recorded using Powerlab/200 and Chart software (AD Instruments).

Cumulative concentration-response curves (CRCs) were constructed for the  $\kappa$ -agonist U50,488 (10<sup>-9</sup>–10<sup>-6</sup> M) and the  $\mu$ -agonist DAMGO (10<sup>-10</sup>–10<sup>-6</sup> M), in the absence and presence of norBNI, 5'-AMN and 5'-MABN (5–250 nM). Following each CRC, tissues were washed until twitches returned to baseline. Agonist CRCs were repeated after incubation with antagonist for 30 min. Agonist responses were calculated as percentage maximal response: (average baseline twitch - average agonist response twitch)/(average baseline twitch - average maximum agonist inhibition of twitch)  $\times$  100. Agonist potency was determined as the negative logarithm of the concentration required to produce 50% of the maximum response ( $pEC_{50}$ ). Agonist  $pEC_{50}$  values in the presence and absence of antagonists were compared using one-way analysis of variance (ANOVA).

Several methods were used to determine antagonist potency (Kenakin, 2009) and curves fitted using GraphPad Prism v5.0 (San Diego, USA). The concentration ratio for the rightward shift of the agonist curve in the presence of antagonist was used to calculate the  $pA_2$  from a Schild linear regression plot. From the CRCs, equiactive concentrations of the agonist were compared in a linear regression and the slope of this regression was used to estimate the  $pK_B$  according to Gaddum's method for measurement of non-competitive antagonist affinity (Kenakin, 2009). Antagonist potency measures were evaluated with one-way ANOVA with repeated measures and Tukey's post hoc test.

### Establishing non-toxic doses in vivo

Drugs, dissolved in 0.9% w/v saline solution, were administered via intraperitoneal injection at volumes of 10 mL/kg (mice) and 1 mL/kg (rats). In vivo studies commenced at 10:00, except the sucrose-consumption test. Toxicity of 5'-AMN and 5'-MABN was assessed in naïve mice using a step-wise minimal numbers approach, starting at a low dose (1 mg/kg) and monitoring behavior (Irwin, 1968). If no toxicity was seen, higher doses up to 20 mg/kg were administered.

### Warm water tail-withdrawal test

Mice were positioned vertically and the tail placed into a beaker of warm water (50°C). The control tail-withdrawal latency was measured 30 min after saline injection (Burke et al., 1994).

Subsequently, the  $\kappa$ -agonist U50,488 (10 mg/kg) was administered and the test latency measured 30 min later. The cut-off time was 15 s. Antinociception was calculated as percentage maximum possible effect (%MPE)=(test latency-control latency)/(15 s-control latency)  $\times$  100. Mice were pre-treated with 0.9 % w/v saline, norBNI, 5'-AMN, or 5'-MABN (1–10 mg/kg) and tail-withdrawal responses measured at 1, 3, 14, 21, 28 and 35 days post-injection.

### K-agonist induced diuresis

Rats were pre-treated with a single injection of 0.9 % w/v saline, norBNI, 5'-AMN or 5'-MABN (1 mg/kg). On test days (1, 8 and 15 days post-injection), the  $\kappa$ -agonist U50,488 (10 mg/kg) was injected to evoke diuresis while control animals received saline. Rats were housed individually in metabolic cages and urine collected (4 h). Rats received two water loads (20 mL/kg) by oral gavage at 1 and 0 h prior to testing.

### Anxiety-related behavior

One group of mice ( $n=70$ ) were used to investigate the effects of norBNI, 5'-AMN and 5'-MABN in the elevated-plus maze (EPM) and the light dark box (LDB) test. Following a single injection of compound (day 0), mice were tested weekly in the EPM (day 7, 14, 21) and LDB (day 8, 15, 22). At least 24 h elapsed between tests and behavior in a particular test was repeated only weekly. Drug-treated mice received norBNI (1–10 mg/kg), 5'-AMN (1 mg/kg) or 5'-MABN (1–10 mg/kg),  $n=10$  per group. Control mice received 0.9% w/v saline. On the test day, one group of mice received diazepam (1 mg/kg), 30 min prior to testing, and all other groups received saline. Animals were handled for 1 week prior to injections and were placed in the behavioral room 1 h prior to testing.

**The EPM test.** Mice were placed in the center of an EPM (EPM2000 Mouse Plus Maze, Campden Instruments) facing an open arm and behavior was recorded for 5 min (Lister, 1987). The time spent in, and entries into, the open arms, and total ambulation were recorded via infrared photobeams and analyzed with Motor Monitor™ software (Campden Instruments). Illumination was 150 lux and <1 lux in the open and closed arms, respectively.

**The LDB test.** Mice were placed at the center of the lit compartment (400 lux), facing the dark compartment and allowed to transition for 10 min between compartments (Crawley, 1985) (Openfield SmartFrame, Campden Instruments). The time spent in, and number of entries into, the lit compartments, and distance travelled in the LDB were recorded via beam-breaks using Motor Monitor™ software (Campden Instruments).

### Depression-related behavior

A separate group of mice ( $n=80$ ) was used to investigate the effects of test compounds using the forced swim test (FST), the tail suspension test (TST) and sucrose consumption. Following a single injection of antagonist (day 0), mice were tested weekly in the FST (day 6, 13, 20), TST (day 7, 14, 21) and sucrose consumption test (day 8, 15, 22). At least 24 h elapsed between tests and behavior in a particular test was repeated only weekly. Mice were treated (day 0) with norBNI (1–10 mg/kg), 5'-AMN (1 mg/kg) or 5'-MABN (1–10 mg/kg),  $n=10$  per group. Control animals



received 0.9% w/v saline injections. On the test day, one group received fluoxetine (10 mg/kg) or U50,488 (5 mg/kg) 30 min prior to behavioral testing, and all other animals received saline. Animals were handled for 1 week prior to injections and placed in the experimental room 1 h prior to testing.

**FST.** Mice were placed in a glass beaker (height 44 cm x diameter 22 cm) filled with water at a depth of 30 cm, at 25±2°C (O'Reilly et al., 2006). Behavior during a 6 min swim session was recorded (Sony DCR-SR52). Analysis of videos was conducted blind to treatment and the time spent climbing, swimming or immobile determined.

**TST.** Mice were suspended by the tail, attached with adhesive tape, from a strain gauge (TS100 Tail Suspension System, Campden Instruments) (Steru et al., 1985). The average force (*N*) produced by mice in the TST apparatus was measured over a 6 min period (Vibration Monitor software, Campden Instruments).

**Sucrose consumption test.** Mice were transferred in their home cages to the procedure room and food/ water deprived for 4 h (from 16.30) (Fukui et al., 2007). Mice were singly housed in test cages for 15 min prior to testing. After acclimatization, bottles containing sucrose solution (5%) were introduced for 1 h. Sucrose consumption was expressed as g sucrose consumed/kg body weight.

### Statistical analysis of behavioral studies

All behavioral data was analyzed using repeated measures one-way ANOVA, followed by Tukey's post hoc test (StatView 5). Values are reported as mean±standard error of the mean (SEM) or mean±95% confidence interval (CI) for each treatment group.

## Results

**[<sup>3</sup>H]-diprenorphine binding assay.** The affinities of 5'-AMN, 5'-MABN, norBNI and GNTI for the κ-, μ- and δ-receptors were determined (Table 1). At the κ-receptor, 5'-MABN had subnanomolar affinity (*K<sub>i</sub>* 0.27±0.08 nM), similar to that of norBNI (*K<sub>i</sub>* 0.29±0.02 nM), whereas 5'-AMN (*K<sub>i</sub>* 1.36±0.98 nM) and GNTI (*K<sub>i</sub>* 0.67±0.18 nM) had somewhat lower affinity. Interestingly, 5'-MABN also showed the greatest affinity for the μ-receptor (*K<sub>i</sub>* 0.88±0.51 nM) while norBNI showed the greatest

affinity for the δ-receptor (*K<sub>i</sub>* 0.46±0.09 nM). Both of the naltrindole derivatives had only modest selectivity for κ/μ (~3–6 fold). 5'-MABN was somewhat selective for κ/δ (~5 fold) while 5'-AMN showed no selectivity.

**[<sup>35</sup>S]-GTPγS assay.** At concentrations up to 10 μM, neither 5'-AMN nor 5'-MABN produced stimulation of [<sup>35</sup>S]-GTPγS binding in C<sub>6</sub>-μ and CHO-κ cell membranes, indicating a lack of any κ- or μ-agonist properties (Supplementary Material, Figure 2). Antagonist activity (*K<sub>e</sub>*) was also determined for 5'-AMN and 5'-MABN at the μ-receptor (11.8±2.7 nM and 0.56±0.26 nM, respectively) and at the κ-receptor (0.32±0.02 nM and 0.06±0.01 nM, respectively). These *K<sub>e</sub>* values suggest that both naltrindole compounds are more potent antagonists at κ than μ-receptors, while 5'-MABN is more potent than 5'-AMN at both μ and κ-receptors.

**Isolated guinea-pig ileum assay.** At maximal doses, the agonists U50,488 and DAMGO produced a ~90% inhibition of electrically-evoked twitches in the guinea-pig ileum. Consistent with reports that norBNI is a reversible competitive antagonist at κ-receptors (Birch et al., 1987), norBNI produced progressive parallel rightward shifts of the U50,488 CRC (control *pEC<sub>50</sub>* = 7.39 (7.73–7.06, 95% CI); 5 nM norBNI, *pEC<sub>50</sub>* = 7.07 (7.19–6.95, 95% CI); *n*=4; *p*< 0.05), without significant effect on *E<sub>max</sub>*, that reversed on washout (Figure 1). Similarly, both 5'-AMN (control *pEC<sub>50</sub>* = 7.48 (7.69–7.27, 95% CI); 20 nM 5'-AMN, *pEC<sub>50</sub>* = 7.27 (7.39–7.15, 95% CI); *n*=4; *p*< 0.05) and 5'-MABN (control *pEC<sub>50</sub>* = 7.19 (7.39–6.99, 95% CI); at 5 nM 5'-MABN, *pEC<sub>50</sub>* = 7.02 (7.08–6.96, 95% CI); *n*=4; *p*< 0.05), produced parallel rightward shifts of the U50,488 CRC (Figure 1). Increasing concentrations of both naltrindole derivatives had no significant effects on U50,488 *E<sub>max</sub>* and the antagonist effects were reversible on wash-out.

Both 5'-AMN (control, *pEC<sub>50</sub>* = 7.72 (8.25–7.04, 95% CI); 80 nM 5'-AMN, *pEC<sub>50</sub>* = 7.38 (7.61–7.14, 95% CI); *n*=4; *p*< 0.05) and 5'-MABN (control *pEC<sub>50</sub>* = 7.68 (8.30–7.06, 95% CI); at 5 nM 5'-MABN, *pEC<sub>50</sub>* = 7.20 (7.22–7.18, 95% CI); *n*=4; *p*< 0.05) produced a significant rightward shift of the DAMGO CRC with no effect on *E<sub>max</sub>*. These μ-antagonist effects were reversible on wash-out.

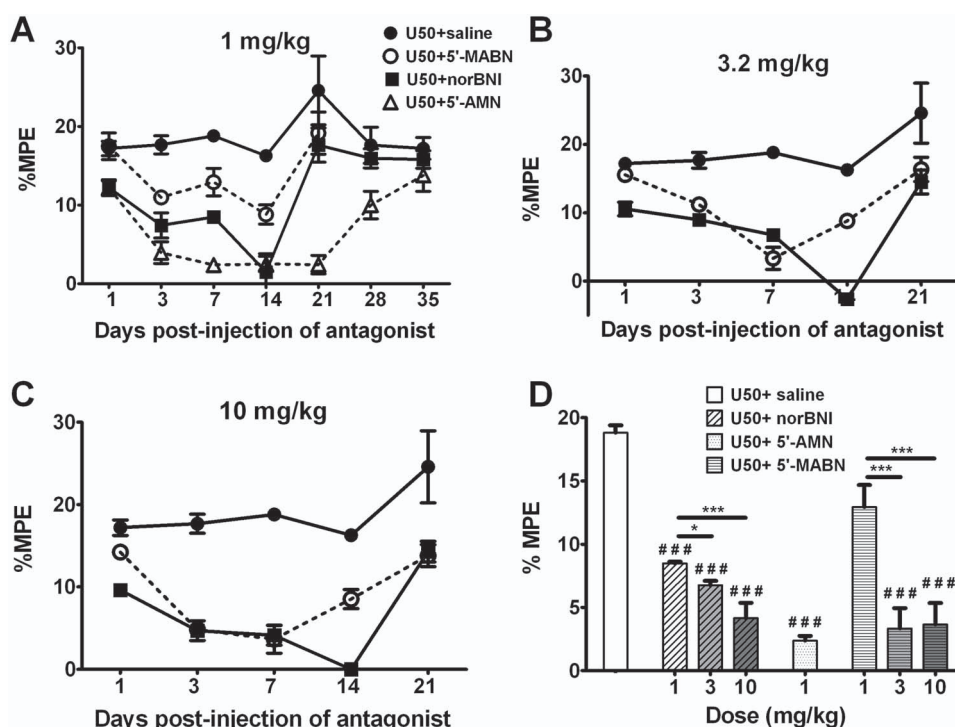
One-way ANOVA with repeated measures revealed no significant differences (*F*<sub>(3, 12)</sub> = 1.09, *p* = 0.39) between the antagonist potency values determined by different methods (Schild plot (Supplementary Material, Figure 2), Gaddum method (Supplementary Material,

**Table 1.** Summary of antagonist affinity and selectivity for κ-, μ- and δ-receptors in the competitive [<sup>3</sup>H]-diprenorphine binding assay.

Ligand	Competitive binding with [ <sup>3</sup> H]-diprenorphine <i>K<sub>i</sub></i> (nM; ±SEM)			κ-selectivity	
	κ	μ	δ	vs μ	vs δ
<b>NorBNI</b>	0.29±0.02	3.99±2.38	0.46±0.09	14	~2
<b>GNTI</b>	0.67±0.18	9.04±1.99	2.86±1.45	~14	~4
<b>5'-AMN</b>	1.36±0.98	7.50±6.45	1.34±2.12	~6	1
<b>5'-MABN</b>	0.27±0.08	0.88±0.51	1.35±1.56	~3	5

5'-AMN: 5'-(2-aminomethyl) naltrindole; 5'-MABN: N-((Naltrindol-5-yl) methyl) pentanimidamide; GNTI: 5'-guanidinonaltrindole; norBNI: norbinaltorphimine; SEM: standard error of the mean.

*K<sub>i</sub>* values for the test ligands were determined by competitive displacement of [<sup>3</sup>H]-diprenorphine binding in CHO-κ, C6-μ and C6-δ cell membranes. Values are the mean±SEM of *n*=2 in triplicate experiments.

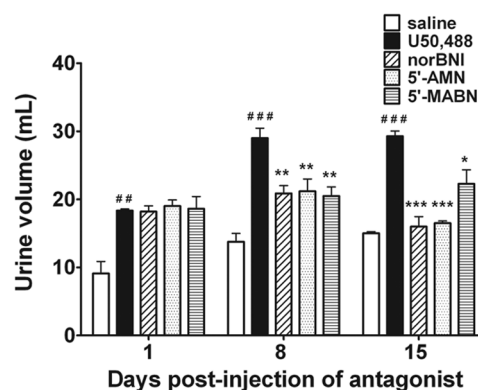


**Figure 2.** Ability of test compounds to block U50,488-induced antinociception in the tail-withdrawal test. Mice received a single injection of saline, norbinaltorphimine (norBNI), 5'-(2-aminomethyl) naltrindole (5'-AMN), or N-((Naltrindol-5-yl) methyl) pentanimidamide (5'-MABN) at doses of (A) 1 mg/kg, (B) 3.2 mg/kg or (C) 10 mg/kg and tail-withdrawal latency measured up to 35 days post-injection. (D) The data at seven days post-injection shows the dose-dependency of antagonism of U50,488-induced antinociception. Data are expressed as mean percentage maximum possible effect (%MPE)  $\pm$  standard error of the mean (SEM),  $n=4$  to 8 per treatment group. Comparison to U50,488 + saline: ### $p<0.001$ . Comparison to 1 mg/kg test compound: \* $p<0.05$ , \*\*\* $p<0.001$ .

Figure 3)) for norBNI, 5'-AMN, and 5'-MABN at both  $\kappa$ - and  $\mu$ -receptors (Table 2). Since Schild's criteria were met, these  $pA_2$  values are cited. Antagonist potencies for each drug were significantly different ( $F_{(4, 12)}=14.23$ ,  $p=0.0002$ ). At the  $\kappa$ -receptor 5'-AMN ( $pA_2$  7.43) was significantly less potent than either norBNI ( $pA_2$  8.30,  $p<0.001$ ) or 5'-MABN ( $pA_2$  8.18,  $p<0.001$ ), which were not significantly different. At the  $\mu$ -receptor 5'-MABN ( $pA_2$  8.18) was significantly more potent than 5'-AMN ( $pA_2$  7.62,  $p<0.05$ ). Together these data indicate that, in the ileum, both naltrindole derivatives are potent reversible competitive antagonists at both  $\kappa$ - and  $\mu$ -receptors.

**Establishing non-toxic doses in vivo.** Intraperitoneal injection in mice of 1–20 mg/kg of 5'-MABN showed no acute toxicity. Low doses of 5'-AMN (1 and 3.2 mg/kg) did not produce any adverse behaviors. However, mice injected with 10 mg/kg 5'-AMN showed abnormal posture and piloerection which returned to normal 1 h post-injection. At 20 mg/kg 5'-AMN caused increased respiratory rate, gasping and convulsions. Mice were immediately killed and autopsy revealed no gross abnormalities. In subsequent in vivo experiments, the maximum dose of 5'-AMN used was 1 mg/kg.

**Warm water tail-withdrawal assay.** NorBNI, 5'-AMN and 5'-MABN significantly reduced the antinociceptive effects of U50,488 (10 mg/kg) (Figure 2). One-way ANOVA with repeated measures revealed significant main effects of treatment ( $F_{(7, 96)}=46.79$ ,



**Figure 3.** Antagonism of U50,488-induced diuresis in rats. Urine output over 4 h, following injection of U50,488 or saline (control) on test day, was measured on days 1, 8 and 15 post-injection of 1 mg/kg norbinaltorphimine (norBNI), 5'-(2-aminomethyl) naltrindole (5'-AMN), or N-((Naltrindol-5-yl) methyl) pentanimidamide (5'-MABN). Data are mean  $\pm$  standard error of the mean (SEM),  $n=4$  per group. Comparisons to U50,488: \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ . Comparisons to saline: ## $p<0.01$ , ### $p<0.001$ .

$p<0.0001$ ) and time of response ( $F_{(4, 96)}=70.45$ ,  $p<0.0001$ ). Furthermore, analysis of treatment by time interactions were identified as significant ( $F_{(24, 96)}=10.09$ ,  $p=0.036$ ).

**Table 2.** Estimates of antagonist potency, derived from isolated guinea pig ileum data, calculated using classical Schild plot, the Gaddum-Schild method, Schild's equation and antagonist affinity ( $pK_B$ ) determined using the Gaddum method.

Ligand; receptor	Slope of Schild plot (+95% CI) <sup>a</sup>	$pA_2$ Schild plot (+95% CI)	$pA_2$ Gaddum-Schild (+95% CI) <sup>b</sup>	$pA_2$ Schild's equation <sup>c</sup>	$pK_B$ Gaddum
<b>NorBNI</b>					
$\kappa$	1.46 (0.32–2.59)	8.30 (10.30–7.72)	8.51 (8.00–9.02)	8.20	8.75
<b>5'-AMN</b>					
$\kappa$	1.16 (0.44–1.87)	7.43 (7.86–7.07)	7.44 (7.77–7.93)	7.45	7.26
$\mu$	1.21 (–0.11–2.55)	7.62 (6.58–9.12)	7.67 (6.93–8.42)	7.33	7.34
<b>5'-MABN</b>					
$\kappa$	0.95 (0.21–1.68)	8.18 (9.81–7.53)	8.09 (7.29–8.89)	8.30	8.43
$\mu$	0.46 (–0.29–1.21)	7.85 (6.58–9.12)	8.09 (6.81–9.38)	8.23	8.94

5'-AMN: 5'-(2-aminomethyl) naltrindole; 5'-MABN: N-((Naltrindol-5-yl) methyl) pentanimidamide; CI: confidence interval; norBNI: norbinaltorphimine. Values expressed are mean $\pm$ 95% CI of  $n=4$  tissues, except where derived as single values.

<sup>a</sup>For Schild plots the regression was linear and the slope was within 95% CI for unity for all antagonists.

<sup>b</sup>Gaddum-Schild model of orthosteric competitive antagonism was used to re-fit the data to a linear model constraining the slope to a value of exactly 1 and the  $pA_2$  determined.

<sup>c</sup>Apparent  $pA_2$  estimates derived from Schild's equation ( $pA_2 = \log(\text{DR}-1) - \log(\text{antagonist})$ ) and the lowest positive  $\log(\text{DR}-1)$  value were calculated from the lowest positive  $\log(\text{DR}-1)$  value that corresponded to a significant rightward shift in the agonist  $pEC_{50}$  in the presence of the antagonist.

Within-treatment comparisons revealed that norBNI (1–10 mg/kg) produced significant blockade of U50,488-induced antinociception at 1, 7 and 14 days post-injection compared to saline-controls ( $p<0.05$ ). Similarly, 5'-MABN (1–10 mg/kg) produced a significant blockade of U50,488-induced antinociception only at 7 and 14 days post-injection ( $p<0.05$ ). A different antagonist time-course was displayed with 5'-AMN (1 mg/kg). While the onset of blockade of U50,488-induced antinociception was evident at one day post-injection ( $p<0.05$ ), it lasted for 28 days ( $p<0.01$ ). Interestingly, the naltrindole derivatives showed comparable potency to norBNI at seven days post-injection (Figure 2(D)). Thus, in vivo, 5'-MABN is equipotent with norBNI and has a similar time course of effect. While, 5'-AMN has a similar potency, it has a significantly longer duration of action than norBNI.

**K-agonist-induced diuresis.** The ability of norBNI, 5'-AMN and 5'-MABN (1 mg/kg) to block U50,488-induced (10 mg/kg) diuresis was examined (Figure 3). One-way ANOVA with repeated measures revealed significant main effects of treatment ( $F_{(4,30)}=26.11, p<0.0001$ ), and time of response ( $F_{(2,30)}=20.58, p<0.0001$ ) on urine output. Furthermore, analysis of treatment by time interactions were identified to be significant ( $F_{(15,30)}=1.97, p=0.05$ ). Within-treatment comparisons revealed that, compared to saline controls, U50,488 produced diuresis on each test day ( $p<0.01$ ). At eight days post-treatment, norBNI, 5'-AMN and 5'-MABN significantly reduced U50,488-evoked diuresis by about 30% ( $p<0.01$ ), an effect that was maintained at 15 days post-treatment. Thus, in vivo, the naltrindole derivatives are as efficacious as norBNI in blocking  $\kappa$ -agonist-induced diuresis.

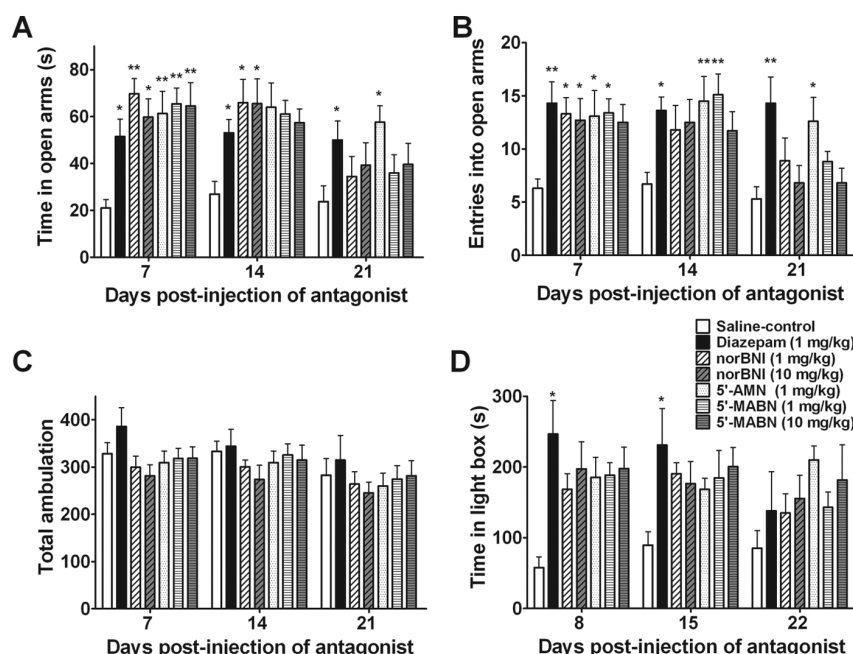
**Effects of 5'-AMN and 5'-MABN on anxiety-related behavior.** Potential anxiolytic responses of the naltrindole derivatives were evaluated in the EPM and LDB (Figure 4). One-way ANOVA with repeated measures of EPM data revealed significant main effects of treatment on the time spent ( $F_{(6,126)}=4.65, p=0.006$ ) and entries into ( $F_{(6,126)}=3.51, p<0.0001$ ) the open arms. Significant main effects of time on the time spent ( $F_{(2,126)}=13.10, p<0.0001$ ), and

entries into ( $F_{(2,126)}=11.73, p<0.0001$ ) the open arms were also found. Furthermore, analysis of treatment by time interactions were identified to be significant for the time spent ( $F_{(12,126)}=1.42, p=0.016$ ) and entries into ( $F_{(12,126)}=2.95, p<0.0001$ ) the open arms. For all drugs, at all doses, total ambulation in the EPM (Figure 4(C)) was not significantly different to saline-controls ( $F_{(6,126)}=1.11, p=0.37$ ), demonstrating an absence of sedative effects.

Within-treatment comparisons to saline treated controls, in the EPM, revealed that the anxiolytic diazepam (1 mg/kg) significantly increased the time spent, and entries into, the open arms on each test day ( $p<0.05$ ). At seven days post-injection, norBNI, 5'-AMN and 5'-MABN also significantly increased the time spent ( $p<0.01$ , Figure 4(A)), and the number of entries into ( $p<0.05$ , Figure 4(B)), the open arms. At 14 days post-injection, norBNI (1 and 10 mg/kg) significantly increased the time spent in the open arms, while 5'-AMN (1 mg/kg) and 5'-MABN (1 mg/kg) significantly increased the entries into the open arms of the EPM, compared with saline-controls ( $p<0.05$ ). Only 5'-AMN significantly increased these same parameters at 21 days post-injection ( $p<0.05$ ), consistent with a longer duration of  $\kappa$ -antagonist activity. Overall, in the EPM, norBNI, 5'-AMN and 5'-MABN exhibited a change in behavior that was consistent with an anxiolytic action.

In the LDB (Figure 4(D)), repeated measures ANOVA revealed a significant effect of drug treatment on the time spent in the lit compartment ( $F_{(6,63)}=2.69, p=0.022$ ). While all drug treatments appeared to increase the time spent in the lit compartment at 8 and 15 days post-injection, only the effects of diazepam were significantly different from saline treated controls ( $p<0.05$ ).

**Effects of 5'-AMN and 5'-MABN on depression-related behavior.** Analysis of behaviors in the FST (Figure 5) with one-way ANOVA with repeated measures revealed significant main effects of treatment on swimming ( $F_{(7,216)}=8.36, p<0.0001$ ), climbing ( $F_{(7,216)}=7.30, p<0.0001$ ) and immobility ( $F_{(7,216)}=15.06, p<0.0001$ ). The analysis also revealed significant main effects of time on swimming ( $F_{(3,216)}=92.92, p<0.0001$ ), climbing



**Figure 4.** Effects of norbinaltorphimine, (norBNI) (1–10 mg/kg), 5'-(2-aminomethyl) naltrindole (5'-AMN) (1 mg/kg) and N-((Naltrindol-5-yl) methyl) pentanimidamide (5'-MABN) (1–10 mg/kg) on anxiety-related behavior in mice. (A) The time spent in open arms, (B) number of entries into the open arms and (C) total ambulation during a 5 min elevated-plus maze (EPM) test are shown. (D) The time spent in the lit compartment during a 10 min light dark box (LDB) test is also shown. Anxiety-related behaviors were measured in the same groups of animals on successive days following a single injection of antagonist, and 30 min after diazepam (1 mg/kg) administration. Data are expressed as mean  $\pm$  standard error of the mean (SEM),  $n=10$  per group. Comparisons to saline: \* $p<0.05$ , \*\* $p<0.01$ .

( $F_{(3, 216)}=62.51$ ,  $p<0.0001$ ) and immobility ( $F_{(3, 216)}=144.70$ ,  $p<0.0001$ ). Furthermore, analysis of treatment by time interactions were identified to be significant for measures of swimming ( $F_{(72, 216)}=2.49$ ,  $p<0.0001$ ), climbing ( $F_{(72, 216)}=1.50$ ,  $p=0.014$ ) and immobility ( $F_{(72, 216)}=2.03$ ,  $p<0.0001$ ).

Post hoc comparisons to saline treated controls revealed that fluoxetine (10 mg/kg), decreased the time spent immobile when tested on days 6 and 13 ( $p<0.01$ ), as expected (Cryan et al., 2005b), whereas U50,488 (5 mg/kg) significantly increased the time spent immobile ( $p<0.01$ ). In pilot studies, 5 mg/kg U50,488 was established to be non-sedating in CD-1 mice (unpublished observations). NorBNI, 5'-AMN and 5'-MABN, significantly decreased the time spent immobile in the FST at 6 and 13 days post-injection ( $p<0.01$ , Figure 5(A)). At 20 and 27 days (data not shown) post-injection there were no significant effects on time spent immobile in the FST. Post hoc analysis of the time spent swimming and climbing revealed few significant effects of drug treatment (Figure 5 (B) and (C)). Overall, in the FST, norBNI, 5'-AMN and 5'-MABN exhibited a change in behavior that was similar to the effects of the antidepressant fluoxetine, whereas the  $\kappa$ -agonist U50,488 demonstrated pro-depressant activity.

In pilot studies, acute administration of U50,488 (5 mg/kg) significantly increased immobility in the TST (a decrease in force), compared with saline-treated controls (control:  $2.2\pm0.16$  N; U50,488:  $0.9\pm0.16$  N,  $p<0.05$ ,  $n=6$  per group), consistent with a pro-depressant effect. Fluoxetine (10 mg/kg) had the opposite effect, significantly reducing immobility (an increase in force), compared to controls (control:  $6.9\pm0.7$  N; fluoxetine:  $7.7\pm0.5$  N,  $p<0.05$ ), consistent with an antidepressant effect (Cryan et al.,

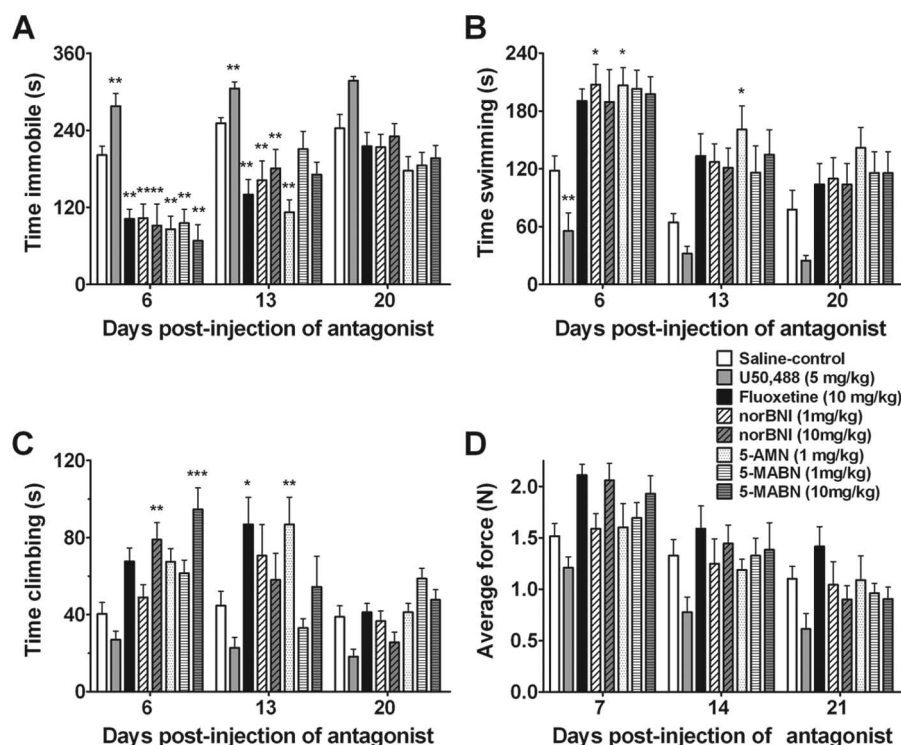
2005a). Analysis of the effects of the naltrindole ligands in the TST (Figure 5(D)) revealed significant main effects of treatment ( $F_{(7, 216)}=5.08$ ,  $p<0.0001$ ), and time ( $F_{(3, 216)}=66.52$ ,  $p<0.0001$ ). Furthermore, analysis of treatment by time interactions were identified to be significant ( $F_{(72, 216)}=2.12$ ,  $p<0.0001$ ). Within-treatment comparisons revealed that while differences were evident between U50,488 treated animals and other drug-treated groups, at each time point no drug treatment group was significantly different from saline-treated controls ( $p>0.05$ ).

Based on (Lewis et al., 2005), in initial experiments, we confirmed that 5% sucrose solution stimulated sucrose consumption in CD-1 mice. Analysis of sucrose consumption data (Figure 6) revealed significant main effects of treatment ( $F_{(7, 144)}=3.86$ ,  $p=0.0013$ ) and time ( $F_{(2, 144)}=14.23$ ,  $p<0.0001$ ). Furthermore, analysis of treatment by time interactions were significant ( $F_{(72, 144)}=2.13$ ,  $p<0.0001$ ). Within-treatment comparisons to saline-treated controls showed that fluoxetine (10 mg/kg) significantly increased sucrose consumption by 40% (control:  $76.8\pm6.0$  g/kg fluoxetine:  $112.6\pm5.1$  g/kg,  $n=10$ ,  $p<0.01$ ). At eight days post-injection, 5'-MABN (10 mg/kg) also significantly increased sucrose consumption ( $p<0.01$ , Figure 6). At 15 and 22 days post-injection, no drug treatment group was significantly different from saline-treated controls ( $p>0.05$ ).

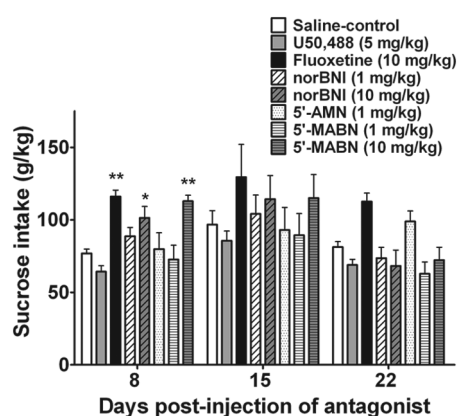
## Discussion

Selective  $\kappa$ -receptor antagonists have been proposed as potential anxiolytic and antidepressant treatments (Bruchas et al., 2010;





**Figure 5.** Effects of norbinaltorphimine, (norBNI) (1–10 mg/kg), 5'-(2-aminomethyl) naltrindole (5'-AMN) (1 mg/kg) and N-((Naltrindol-5-yl) methyl) pentanimidamide (5'-MABN) (1–10 mg/kg) on depression-related behavior in mice. (A) The time spent immobile, (B) swimming and (C) climbing during a 6 min forced swim test (FST) is shown. (D) In the tail suspension test (TST), a decrease in average force corresponds to increased immobility. Depression-related behaviors were measured in the same groups of animals on successive days following a single injection of antagonist, and 30 min after U50,488 (5 mg/kg) or fluoxetine (10 mg/kg) administration. Data are expressed as mean  $\pm$  standard error of the mean (SEM),  $n=10$  per group. Comparisons to saline: \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$



**Figure 6.** Effects of norbinaltorphimine, (norBNI) (1 and 10 mg/kg), 5'-(2-aminomethyl) naltrindole (5'-AMN) (1 mg/kg) and N-((Naltrindol-5-yl) methyl) pentanimidamide (5'-MABN) (1 and 10 mg/kg) on sucrose consumption in mice. Responses were measured at weekly intervals after a single injection of antagonist, and 30 min after U50,488 (5 mg/kg) or fluoxetine (10 mg/kg) administration. The mean  $\pm$  standard error of the mean (SEM) sucrose (5%) consumed in g of sucrose per kg body weight, during a 1 h test, is shown.  $n=10$  per treatment group. Comparisons to saline: \* $p<0.05$ , \*\* $p<0.01$

Knoll and Carlezon, 2010). We have characterized the  $\kappa$ -antagonist activity of two naltrindole-derived ligands in vivo for the first time. Systemic administration of both 5'-AMN and 5'-MABN decreased anxiety- and depression-related behaviors in the EPM and FST respectively. In vitro, these compounds displayed significant  $\mu$ -antagonist activity; however, concurrent  $\mu$ -receptor antagonism did not negate the anti-depressant effects of  $\kappa$ -receptor antagonism. Indeed, both 5'-AMN and 5'-MABN were as effective as the standard selective  $\kappa$ -antagonist norBNI.

This is also the first study to examine the effects of  $\kappa$ -antagonists on anxiety- and depression-related behaviors in the outbred CD-1 mouse strain. Previous studies of  $\kappa$ -receptor involvement in these behaviors have largely focused on the inbred C57Bl/6 strain (McLaughlin et al., 2003; McLaughlin et al., 2006b; Wittmann et al., 2009). Typically inbred strains are used in behavioral studies to reduce the variability in data and to facilitate the identification of genetic factors influencing mood (Jacobson and Cryan, 2007). It has been reported that CD-1 mice are relatively insensitive to fluoxetine in the FST and to fluvoxamine in the TST (Lucki et al. 2001; Van der Heyden et al. 1987). In our experiments with CD-1 mice we were able to show that baseline immobility in the FST or TST was not low and that acute fluoxetine (10 mg/kg) significantly decreased the time spent immobile in the FST and TST (in pilot studies); although this latter effect was not significant

in the full study (Figure 5). Our observations in the outbred CD-1 strain add weight to the involvement of  $\kappa$ -opioid systems in depression- and anxiety-related behaviors.

Contrary to our hypothesis, the primary amine group in 5'-AMN did not produce a shorter-acting  $\kappa$ -antagonist. Both naltrindole-derived ligands produced significant blockade of U50,488-induced antinociception in the tail-withdrawal assay, with comparable potency to norBNI, demonstrating their  $\kappa$ -antagonist action in vivo for the first time. However, 5'-AMN blocked U50,488-induced antinociception at 28 days post-injection, indicating a longer duration of action than either norBNI or 5'-MABN. The long-lasting actions of  $\kappa$ -antagonists have been proposed to be due to the lipophilic nature of these ligands, resulting in them being deposited in lipid bilayers and causing sustained release in the brain, or due to metabolism to long-lasting metabolites (Horan et al., 1992). Others have proposed inverse agonist actions (Hampson et al., 2000; Mizoguchi et al., 2002; Simen and Miller, 1998; Wang et al., 2007). More recently, it has been proposed that ligand-directed signaling occurs at the  $\kappa$ -receptor (Bruchas and Chavkin, 2010). In this model, the long duration of action of  $\kappa$ -antagonists, such as norBNI, can be attributed to their ability to activate JNK phosphorylation which in turn leads to long-term inactivation of the  $\kappa$ -receptor (Bruchas and Chavkin, 2010; Melief et al., 2010; Melief et al., 2011). Which of these mechanisms may account for the increased duration of  $\kappa$ -antagonism produced by 5'-AMN is unknown.

The duration of anxiolytic- and antidepressant-like effects was comparable to that seen in the tail-withdrawal assay, although generally not as long-lasting. For example, 5'-AMN continued to demonstrate significant anxiolytic activity in the EPM at 21 days post-injection while norBNI and 5'-MABN did not. Furthermore, the behavioral effects produced by norBNI, 5'-AMN and 5'-MABN in this study were still apparent up to 13 days post-injection in the FST. It has been argued that the long-lasting duration of  $\kappa$ -antagonist activity was required for its behavioral effects. NorBNI (20 mg/kg) induced antidepressant-like effects in the rat FST which lasted up to four weeks post-injection (Wiley et al., 2009), whereas others have shown the effects to be transient and not apparent at 3–14 days post-injection (Zhang et al., 2007). However, short-acting  $\kappa$ -antagonists (<7 days) have recently been shown to produce significant anxiolytic-like effects in the rat EPM (Peters et al., 2011).

We have also examined the effects of  $\kappa$ -receptor agonists/antagonists for the first time on sucrose consumption in mice as a model of anhedonia. Although sucrose consumption in an acute test situation is often used as a measure of depression-related behavior, it has only been validated in the context of the chronic mild stress model to our knowledge, where fluoxetine reverses stress-induced anhedonia (Muscat et al., 1992). However, in our model we demonstrated that fluoxetine significantly increased sucrose consumption in an acute sucrose consumption test situation and we were able to detect a similar antidepressant-like effect with 10 mg/kg 5'-MABN at eight days post-injection. However, this was not evident for any other treatment groups, suggesting that the sucrose consumption paradigm may not be robust enough for detecting the antidepressant effects of  $\kappa$ -antagonists. In rats, the consumption of concentrated sucrose solutions (e.g. 10%) is stimulated by U50,488 treatment, whereas there is no effect on low sucrose concentration solutions (e.g. 2.5%) (Lynch and Burns,

1990; Ruegg et al., 1997). Furthermore, norBNI treatment in rats had no effect on sucrose (0.5%) consumption but did reverse the effects of U50,488 (Wiley et al., 2009). While we did not examine a range of sucrose concentrations, we did show that 5% sucrose stimulated consumption, compared to water alone, in CD-1 mice. Alternatively, the sucrose consumption model may not adequately stress the mice to activate the dynorphin- $\kappa$ -opioid receptor system. The importance of acute stress in inducing dynorphin release and endogenous  $\kappa$ -receptor activation is well-documented (Schwarzer, 2009; Tejada et al., 2012). In previous studies of the effects of  $\kappa$ -antagonists on depression- and anxiety-related behavior, the antagonists were administered prior to, or immediately following, stress (McLaughlin et al., 2003; McLaughlin et al., 2006b; Wittmann et al., 2009). For example, Wittmann et al. (2009) demonstrated  $\kappa$ -receptor involvement in behaviors in the FST only after repeated forced swim sessions. However, in our experiments, we were able to demonstrate significant  $\kappa$ -antagonist effects without first stressing the mice in the FST, but not in the TST or sucrose consumption model. Similarly, the absence of additional stressors may also account for an apparent anxiolytic effect of  $\kappa$ -antagonists in the more aversive EPM, compared to the LDB.

Both 5'-AMN and 5'-MABN have been shown previously to have promising selectivity for the  $\kappa$ -receptor in vitro (Olmsted et al., 1993; Stevens et al., 2000). Olmsted et al., (1993;  $K_i$   $\kappa$ -receptor  $0.30 \pm 0.2$ ) reported that 5'-MABN showed a  $\kappa/\mu$ -selectivity of 1000-fold. In contrast, we have shown that the substitution of a methyl amine group (5'-AMN) with a (2-butylamidino) methyl group (5'-MABN) on the 5'-position of the naltrindole core increased the affinity of 5'-MABN over 5'-AMN at both  $\kappa$ - and  $\mu$ -receptors (Table 1). Interestingly, in the literature there is a wide variation in the reported  $K_i$  values for norBNI at  $\kappa$ -receptor (0.09 to 1.09 nM) and at the  $\mu$ -receptor (1.2 to 101.9 nM) and the values we obtained for norBNI are well within these ranges. The apparent high affinity of the naltrindole-derived compounds for both  $\kappa$ - and  $\mu$ -receptors was reflected in guinea-pig ileum studies, where 5'-MABN was equipotent with norBNI and more potent than 5'-AMN at  $\kappa$ -receptors, but both naltrindole-derived compounds were moderately potent  $\mu$ -receptor antagonists. Surprisingly, the  $pA_2$  values obtained in the guinea-pig ileum (Table 2) indicated ~10-fold lower affinity than the  $K_i$  values obtained with [ $^3$ H]-diprenorphine binding. One explanation, given the slow kinetics of these molecules, is that the 30 min incubation time was insufficient to achieve antagonist equilibrium in the guinea-pig ileum.

Given the significant  $\mu$ -receptor antagonist activity of the naltrindole-derived compounds, it is perhaps surprising that we were able to detect any antidepressant- or anxiolytic-like responses. Endorphins are euphorogenic and have long been proposed to have antidepressant actions (Berrocoso et al., 2009; Emrich et al., 1983). Buprenorphine, a  $\kappa$ -antagonist with partial  $\mu$ -receptor agonist activity, administered alone, or in combination with the opioid receptor antagonist naltrexone to enhance its  $\kappa$ -antagonism, has been shown to have antidepressant effects in patients (Emrich et al., 1982; Rothman et al., 2000).  $\mu$ -receptors have also been implicated in the actions of antidepressants like venlafaxine, its antidepressant-like effects are abolished in  $\mu$ -receptor knockout mice (Ide et al., 2010). Here, we have shown antidepressant- and anxiolytic-like effects for two naltrindole-derived ligands that are potent antagonists at both  $\kappa$ - and  $\mu$ -receptors, suggesting that  $\kappa$ -selectivity might not be as crucial for such activity as previously thought.

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## Conflicts of interest

The authors declare that there are no conflict of interest.

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