

## Naloxone benzoylhydrazone activates Extracellular Signal-Regulated Protein Kinases and modulates Nociceptin Opioid Peptide Receptor activity

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**Abstract:** Naloxone benzoylhydrazone (NalBzoH) is often used as a nociceptin opioid peptide (NOP) receptor antagonist. However, NalBzoH is also a mixed  $\mu$  antagonist/ $\kappa$  and  $\delta$  opioid agonist and its actions at the NOP receptor range from an antagonist to partial agonist, depending on the effector response/system studied. We now report that NalBzoH activates endogenously expressed  $\kappa_3$  opioid, but not NOP, receptors to stimulate extracellular signal-regulated protein kinases 1/2 and, subsequently, to mediate changes in the expression of tyrosine hydroxylase and G protein-coupled receptor kinase 2 in SH-SY5Y human neuroblastoma cells. Furthermore, pretreatment with NalBzoH produces homologous desensitization of the  $\kappa_3$  opioid receptor as well as heterologous desensitization of the NOP receptor. In contrast, pretreatment with the NOP agonist, orphanin FQ/nociceptin (OFQ/N), desensitizes only NOP but not the NalBzoH response, suggesting the involvement of a receptor, distinct from NOP, in mediating the actions of NalBzoH in SH-SY5Y cells.

**Keywords:** orphanin FQ/nociceptin, tyrosine hydroxylase, G protein-coupled receptor kinase 2

### INTRODUCTION

The multiplicity of opioid receptors and their widespread distribution throughout the central and peripheral nervous systems enables opiates and endogenous opioid peptides to elicit a broad spectrum of pharmacological and physiological actions. While cloning studies provide a general classification of opioid receptors into  $\mu$ ,  $\delta$ ,  $\kappa$  and nociceptin opioid peptide (NOP) receptors, pharmacological evidence suggests the existence of subtypes for these cloned receptors<sup>[1,2]</sup>. The  $\kappa$  opioid receptor has been classified into three subtypes:  $\kappa_1$ ,  $\kappa_2$  and  $\kappa_3$ . Studies elucidating the actions of NalBzoH at classical opioid receptor subtypes described it as a mixed  $\kappa$  agonist/ $\mu$  antagonist<sup>[3-7]</sup>, as a  $\delta$  opioid receptor antagonist with partial agonist actions at the  $\kappa_1$  opioid receptor<sup>[5]</sup>, and as an agonist at recombinant and olfactory  $\delta$  opioid receptors<sup>[8,9]</sup>. Besides demonstrating a potent antagonistic action of NalBzoH at  $\mu$  opioid receptors, the initial in vivo studies revealed that NalBzoH was an agonist at a novel  $\kappa$  opioid receptor and produced analgesia when administered into the cerebral ventricles. Like other opioid agonists, NalBzoH also inhibited forskolin-stimulated cAMP accumulation, and this was not reversed by selective  $\mu$ -,  $\delta$ - or  $\kappa_1$ -receptor antagonists<sup>[10,11]</sup> or by antisense DNA targeting the NOP receptor<sup>[12]</sup>. It was, however, reversed by a less

selective  $\mu/\kappa$  antagonist, Mr2266<sup>[10,12]</sup>. Likewise, NalBzoH-mediated analgesia was not reversed by selective  $\mu$ -,  $\delta$ - or  $\kappa_1$ -receptor antagonists, and failed to exhibit any cross-tolerance with  $\mu$ - or  $\kappa_1$ -agonists<sup>[3,4]</sup>. These data collectively suggested that NalBzoH activates a novel  $\kappa$ -receptor subtype,  $\kappa_3$ , which corresponds to the nalorphine (N) receptor proposed by Martin<sup>[13]</sup>.

While the molecular characterization of the  $\kappa_3$  receptor is not complete, our understanding of the pharmacological actions of NalBzoH has increased. A study performed with NOP receptor-knockout mice suggests that NalBzoH elicits its analgesic response by antagonizing the hyperalgesic action of the endogenous peptide, OFQ/N, at the NOP receptor and it was proposed that the  $\kappa_3$  receptor was the NOP receptor itself<sup>[14,15]</sup>. Several other studies supported the role of NalBzoH as a competitive antagonist at the NOP receptor<sup>[14,16,17]</sup>, albeit with less potency than its antagonist actions at the  $\mu$  receptor<sup>[6,18-22]</sup>. However, other studies strongly negated this relationship. In both rats and mice, an approach using antisense DNA clearly demonstrated that the  $\kappa_3$  receptor is not identical to NOP, but rather shares a common sequence with it<sup>[23-27]</sup>. Recently, we illustrated differences between NOP and  $\kappa_3$  opioid receptors in BE(2)-C human neuroblastoma cells<sup>[12]</sup>.

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To further distinguish the actions of NalBzoH from those of OFQ/N at the cellular level, we examined the activation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) by  $\kappa_3$  and NOP receptors endogenously expressed<sup>[11,28-30]</sup> in SH-SY5Y human neuroblastoma cells and their resulting effects on levels of tyrosine hydroxylase (TH) and G protein-coupled receptor kinase (GRK) 2.

## MATERIALS AND METHODS

**Materials:** The following drugs and materials were purchased from, or kindly provided by, the sources indicated: cell culture media (GibcoBRL, Grand Island, NY); fetal bovine sera, penicillin G and streptomycin sulfate (Atlanta Biologicals, Norcross, GA); NalBzoH and Mr2266 (Dr. Gavril W. Pasternak, Memorial Sloan-Kettering Cancer Center, New York, NY); OFQ/N, Naltriben, DPDPE and CTAP (Research Technology Branch of National Institute on Drug Abuse, Rockville, MD); peptide III BTD (Multiple Peptide Systems, San Diego, CA); PTX (List Biological Laboratories, Campbell, CA); chelerythrine chloride and wortmannin (Research Biochemicals, Inc., Natick, MA); PD98059, phospho-specific ERK1/2, and ERK1/2 antisera (Cell Signaling Technology, Inc., Beverly, MA); [<sup>3</sup>H]cAMP (Amersham Life Sciences, Arlington Heights, IL); TH and GAPDH antibodies (Chemicon International, Inc., Temecula, CA); GRK2 antisera (sc-562; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). All SDS-PAGE reagents were obtained from Bio-Rad (Hercules, CA) and other chemicals/reagents were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell culture and experimental procedures:** SH-SY5Y neuroblastoma cells (passages 38-52) were cultured and maintained as described<sup>[11]</sup>. Cells were grown to 60-80% confluence in 24-well or 100 cm<sup>2</sup> dishes in a 6% CO<sub>2</sub>-94% air humidified atmosphere at 37°C. ERK1/2 activation following short-term agonist exposure or changes in TH and GRK2 proteins after prolonged agonist exposure were measured as described<sup>[30,31]</sup>. The ability of NalBzoH or OFQ/N to inhibit forskolin-stimulated cAMP accumulation was measured following drug pretreatment<sup>[10]</sup>, wherein cells were washed in serum-free media and pretreated for 1 h at 37°C with the indicated drug in the same media containing protease-free bovine serum albumin (0.1%) and bacitracin (0.25 mg/ml). NalBzoH was dissolved in 25 mM Tris Citrate, pH 5.

**Image and statistical analysis:** Following SDS-PAGE, immunoreactive bands for ERK, TH, GRK2 and GAPDH were densitized as described<sup>[30,31]</sup>. Phospho/total ERK, TH/GAPDH and GRK2/GAPDH immunoreactivity (IR) ratios were calculated for each band (for statistical analysis) and normalized with respect to basal values (for data presentation). Representative immunoblots were scanned (Hewlett

Packard Scanjet 6300C, with 1200 dpi optical resolution); the resulting images were cropped and sized for figures using Adobe Photoshop, version 5.0 for PC. Concentration-response curves were fitted and log EC<sub>50</sub> values were determined using non-linear regression analysis (GraphPad Prism version 4.00 for Windows; GraphPad Software, San Diego, CA). Statistical comparisons of data were performed with unpaired *t* test or one-way ANOVA followed by Dunnett's or Tukey's post-hoc test, where appropriate, using GraphPad Prism. Data are expressed as mean ± s.e.m. unless otherwise indicated and differences were considered significant if  $p \leq 0.05$ .

## RESULTS

**Time- and concentration-dependent activation of ERK1/2 by NalBzoH:** SH-SY5Y cells were stimulated with NalBzoH (1 μM) for various time periods ranging from 1-60 min and cell lysates were subjected to SDS-PAGE as described in Methods to determine ERK1/2 activation. Maximal ERK1/2 activation (3.1 fold) was achieved in 5 min (Fig. 1A); phospho/total ERK immunoreactivity (IR) ratios for ERK1 and ERK2 were  $3.97 \pm 0.29$  and  $3.06 \pm 0.23$  with NalBzoH versus basal ratios of  $1.29 \pm 0.3$  and  $0.99 \pm 0.23$ , respectively.

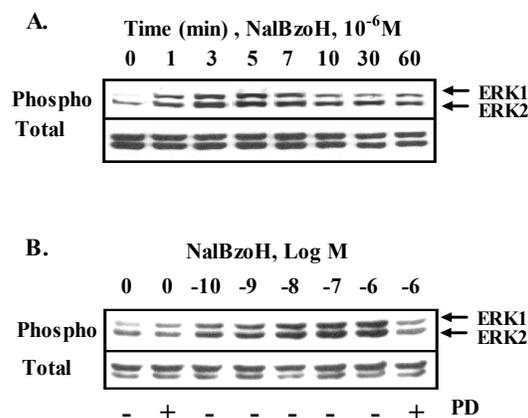


Fig. 1: Time- and concentration-dependent stimulation of ERK1/2 by NalBzoH: Cells were serum-deprived for 24 h and (A) incubated with 1 μM NalBzoH for the indicated time or (B) were pretreated with or without PD98059 (PD; 10 μM) for 60 min followed by a 5 min stimulation with increasing concentrations of NalBzoH as described in Methods. Cell lysates were subjected to SDS-PAGE and membranes were sequentially blotted with phospho-specific ERK1/2 and ERK1/2 antisera. Immunoblots are representative of four to six independent experiments.

Significant ERK1/2 activation persisted for 10 min; thereafter gradually decreasing to 1.4 fold of basal

levels by 60 min of NalBzoH treatment. Based on this time course study, all subsequent experiments of the acute ERK1/2 activation by NalBzoH were for 5 min. The concentration-response curves for ERK1/2 activation by NalBzoH reveal an appreciable increase in ERK1/2 activity with drug concentrations as low as 1 nM and maximal activation by 1  $\mu$ M (Fig. 1B). The Log EC<sub>50</sub> values for ERK1 and ERK2 activation are  $-7.96 \pm 0.16$  and  $-8.19 \pm 0.22$ , respectively, indicating that NalBzoH activates both isoforms with similar potency. NalBzoH stimulation of ERK1/2 was completely abolished upon pretreatment with PD98059 (10  $\mu$ M; Fig. 1B), an inhibitor of mitogen-activated protein kinase kinases 1 and 2 (MEK1/2); immediate upstream activators of ERK1/2. No significant change in total ERK levels was observed with any treatment.

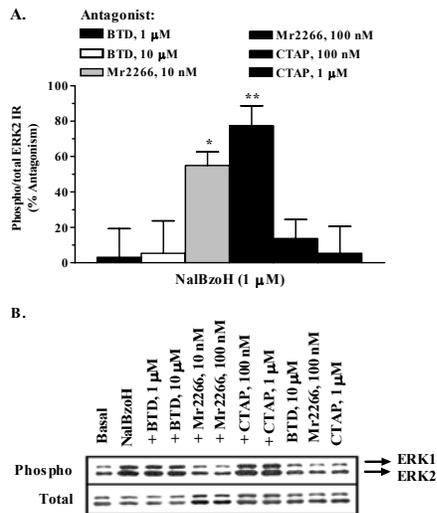


Fig. 2: Antagonism of NalBzoH-mediated ERK1/2 activation. Cells were serum-deprived for 24 h, washed and treated for 5 min with the indicated concentrations of peptide III BTB (BTB), Mr2266 or CTAP before ERK1/2 stimulation with 1  $\mu$ M NalBzoH for 5 min. Cell lysates were subjected to immunoblot analysis using phospho-specific ERK1/2 and ERK1/2 antisera as described in Methods. **A.** Data are compiled by densitometric analysis and represent the mean percentage antagonism of NalBzoH-mediated ERK2 activation ( $2.80 \pm 0.09$  fold)  $\pm$  s.e.m. values from four independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ ; significantly different from NalBzoH-mediated ERK2 activation. **B.** A representative immunoblot.

**NalBzoH stimulates a receptor distinct from NOP or  $\mu$  opioid receptors to activate ERK1/2:** Among the receptors with which NalBzoH has been found to interact,  $\mu$ ,  $\delta$ ,  $\kappa_3$  and NOP are present in SH-SY5Y cells [11,29,32,33]. Therefore, to determine the receptor/s involved in NalBzoH-mediated ERK1/2 stimulation in

this cell line, cells were pretreated with or without peptide III BTB (NOP receptor antagonist<sup>[34]</sup>), CTAP ( $\mu$  receptor antagonist<sup>[11]</sup>) or Mr2266 (nonselective,  $\mu$  and  $\kappa$  receptor antagonist<sup>[10,35]</sup>) prior to NalBzoH challenge. NalBzoH-stimulated ERK1/2 phosphorylation was not antagonized by peptide III BTB or CTAP (Fig. 2). Mr2266, previously shown to block NalBzoH-mediated inhibition of cAMP accumulation<sup>[10,12]</sup>, significantly blocked the ERK1/2 activation induced by NalBzoH in the present study as well (Fig. 2). The antagonists alone did not alter the basal ERK1/2 phosphorylation levels at the concentrations used (Fig. 2B).

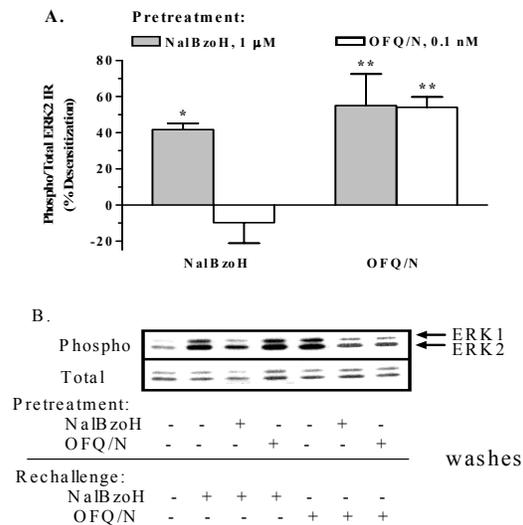


Fig. 3: Effect of agonist pretreatment on subsequent ERK1/2 stimulation by NalBzoH or OFQ/N. SH-SY5Y cells were serum-deprived for 24 h, washed free of media and pretreated for 5 min with 1  $\mu$ M NalBzoH ( $\kappa_3$  agonist) or 0.1 nM OFQ/N (ORL1 agonist). Cells were washed with ice-cold HBSS and re-challenged with NalBzoH (1  $\mu$ M) or OFQ/N (1 pM) for 5 min at 37°C. Cell lysates were subjected to SDS-PAGE and membranes were immunoblotted with phospho-specific ERK1/2 and ERK1/2 antisera. **A.** Data are compiled by densitometric analysis and are presented as mean percentage inhibition of agonist-induced ERK2 activation  $\pm$  s.e.m. values from three to four separate experiments. \* $p < 0.05$ , \*\* $p < 0.01$ ; significantly different from agonist-mediated ERK2 activation. **B.** A representative immunoblot.

A receptor desensitization approach was utilized to further explore the site of action of NalBzoH. Cells were pretreated with maximally effective concentrations of NalBzoH (1  $\mu$ M<sup>[10,12]</sup>) or OFQ/N (0.1 nM<sup>[29-31]</sup>) for 5 min, washed and re-challenged to determine ERK1/2 activation. Stimulating cells with the NOP receptor agonist, OFQ/N (1 pM), for 5 min induced a  $2.66 \pm 0.09$  fold activation of ERK2 as reported earlier<sup>[30]</sup>; NalBzoH induced a  $2.71 \pm 0.11$  fold activation of ERK2. Pretreatment with NalBzoH or

OFQ/N significantly inhibited the subsequent stimulation of ERK1/2 upon rechallenge with the same drug (Fig. 3), demonstrating that each drug was able to induce homologous desensitization of its own ERK1/2 response. Pretreating the cells with NalBzoH also produced a significant desensitization of OFQ/N-mediated ERK1/2 activation (Fig. 3). However, pretreatment with OFQ/N had no effect on NalBzoH-mediated ERK1/2 activation (Fig. 3). This further suggests that NalBzoH acts at a site distinct from that stimulated by OFQ/N.

The inhibitory actions of NalBzoH and OFQ/N on adenylyl cyclase also were examined to confirm the interactions suggested by the ERK1/2 activation experiments. Cells were stimulated with NalBzoH (1  $\mu$ M) or OFQ/N (0.1 nM) for 1 h, washed extensively, and rechallenged for 10 min with increasing concentrations of NalBzoH or OFQ/N to test their ability to inhibit forskolin (10  $\mu$ M)-stimulated cAMP accumulation. Similar to our previous observation, NalBzoH pretreatment desensitized both OFQ/N- and NalBzoH-mediated inhibition of cAMP accumulation, reducing the maximal response to each by >50% (Fig. 4). However, pretreatment with OFQ/N desensitized only subsequent OFQ/N-mediated inhibition of cAMP accumulation (by 58%; Fig. 4). Interestingly, OFQ/N pretreatment significantly increased the potency of NalBzoH to inhibit cAMP accumulation in SH-SY5Y cells as indicated by EC<sub>50</sub> shift from 30 nM to 3 nM ( $p < 0.0001$ ; Fig. 4).

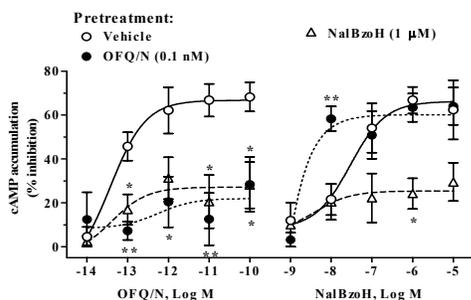


Fig. 4: Effect of agonist pretreatment on subsequent OFQ/N- or NalBzoH-mediated inhibition of cAMP accumulation. Cells were incubated for 1 h with vehicle or with indicated concentrations of OFQ/N or NalBzoH as described in Methods. Intact cells were then assayed for 10 min with increasing concentrations of OFQ/N or NalBzoH to inhibit forskolin (10  $\mu$ M)-stimulated cAMP accumulation. Data represent mean  $\pm$  s.e.m. values from four to five independent experiments performed in duplicate. The Log EC<sub>50</sub> values for inhibiting forskolin-stimulated cAMP accumulation were  $-13.43 \pm 0.09$  (OFQ/N) and  $-7.53 \pm 0.15$  (NalBzoH) in vehicle-treated cells. \* $p < 0.05$ , \*\* $p < 0.01$ ; significantly different from agonist-mediated inhibition of forskolin-stimulated cAMP accumulation in vehicle-treated cells.

**NalBzoH activates a distinct signaling cascade as compared to OFQ/N for stimulating ERK1/2:** To examine the signaling components involved in NalBzoH- and OFQ/N-mediated ERK1/2 activation,

cells were pretreated with 100 ng/ml pertussis toxin (PTX) for 24 h, 1  $\mu$ M chelerythrine chloride for 5 min or 100 nM wortmannin for 10 min prior to agonist challenge. PTX completely attenuated the ERK1/2 signaling by NalBzoH and OFQ/N, indicating a role for G<sub>i/o</sub> proteins in these responses (Fig. 5). The selective protein kinase C (PKC) inhibitor, chelerythrine, blocked the ERK1/2 activation elicited by NalBzoH and OFQ/N by 54%, while the selective phosphatidylinositol 3-kinase (PI3K) inhibitor, wortmannin, blocked only the OFQ/N response (by 48%; Fig. 5). None of the inhibitors alone had any effect on basal ERK1/2 activation at the concentrations used (Fig. 5B). Therefore, while NalBzoH-mediated ERK1/2 signaling partly involves the activation of PKC, OFQ/N-stimulated signaling is mediated by PKC and PI3K. These results suggest that the actions of OFQ/N involve intracellular signaling cascades distinct from those of NalBzoH.

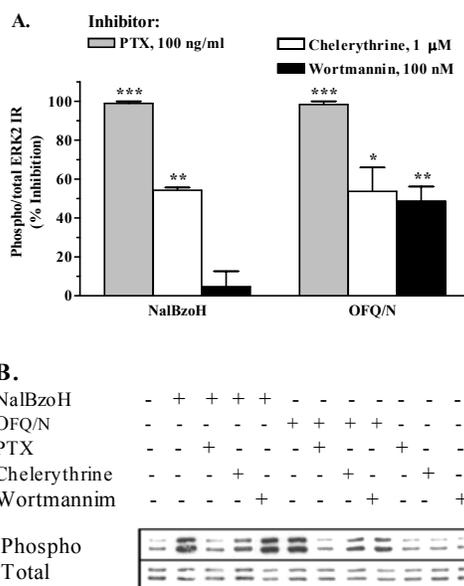


Fig. 5: Blockade of  $\kappa_3$  or ORL1 receptor-mediated ERK1/2 activation by pertussis toxin and protein kinase inhibitors. SH-SY5Y cells were serum-deprived for 24 h in the presence or absence of the indicated concentration of pertussis toxin. Cells were washed and pertussis toxin-naive cells were treated with or without the indicated concentrations of chelerythrine (PKC inhibitor) for 5 min or wortmannin (PI3K inhibitor) for 10 min prior to ERK1/2 stimulation by NalBzoH (1  $\mu$ M) or OFQ/N (1 pM). Cell lysates were subjected to SDS-PAGE and membranes were immunoblotted with phospho-specific ERK1/2 and ERK1/2 antisera. **A.** Data are compiled by densitometric analysis and are presented as mean percentage inhibition of agonist-induced ERK2 activation (NalBzoH:  $3.16 \pm 0.19$  and OFQ/N:  $2.95 \pm 0.14$  fold)  $\pm$  s.e.m. values from four independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; significantly different from agonist-mediated ERK2 activation. **B.** A representative immunoblot.

**Effect of prolonged NalBzoH exposure on endogenous TH and GRK2 levels in SH-SY5Y cells:**

We have previously demonstrated that prolonged stimulation (24 h) of SH-SY5Y cells with  $\mu$  agonists upregulates endogenous TH levels while stimulation with OFQ/N downregulates TH levels<sup>[30]</sup>. Prolonged treatment with morphine or OFQ/N also upregulates endogenous GRK2 levels via activation of ERK1/2<sup>[31]</sup>. Therefore, we studied the effects of prolonged NalBzoH treatment on TH and GRK2 levels in SH-SY5Y cells. Cells were treated with NalBzoH (1  $\mu$ M) for 24 h and cell lysates were subjected to SDS-PAGE to detect TH and GRK2 levels. Prolonged NalBzoH exposure significantly increased the levels of TH (Fig. 6) and GRK2 (Fig. 7); this was blocked upon inclusion

of the  $\mu/\kappa$  receptor antagonist, Mr2266, but not the NOP receptor antagonist, peptide III BTd (Figs. 6 and 7). In contrast, OFQ/N-mediated GRK2 upregulation was reversed by peptide III BTd, but not Mr2266 (Fig. 7). As a control to determine if activation of  $\delta$  opioid receptors stimulates upregulation of GRK2 and TH in SH-SY5Y cells, cells were treated with the full  $\delta$  opioid agonist, DPDPE (1  $\mu$ M) for 24 hr. Treatment with DPDPE failed to significantly increase levels of GRK2 ( $108.4 \pm 13.6\%$  Basal;  $n=5$ ) or TH ( $100.4 \pm 19.6\%$  Basal;  $n=5$ ).

Treating the cells with NalBzoH in the presence of PD98059 (10  $\mu$ M) completely abolished NalBzoH-induced upregulation of both, TH and GRK2 (Figs. 6 and 7), indicating the involvement of ERK1/2 in both events. Neither PD98059 nor the inhibitors alone had a significant effect on basal TH or GRK2 levels (Figs. 6B and 7B).

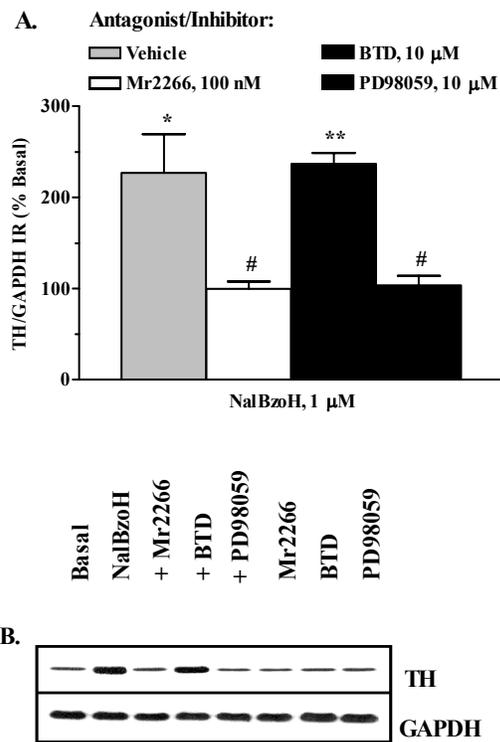


Fig. 6: TH induction following prolonged activation of the  $\kappa_3$  receptor is mediated via ERK1/2. Cells were pretreated with or without indicated concentrations of Mr2266 or BTD for 5 min or PD98059 for 60 min prior to stimulation with 1  $\mu$ M NalBzoH for 24 h as described in Methods. Cell lysates were immunoblotted with TH and GAPDH antibodies. **A.** Densitometric analysis of immunoreactive bands represents the mean percentage increase  $\pm$  s.e.m. values over basal TH/GAPDH ratio ( $0.86 \pm 0.35$ ) from four to six independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ ; significantly different from basal and # $p < 0.05$ ; significantly different from NalBzoH-induced TH levels. **B.** A representative immunoblot.

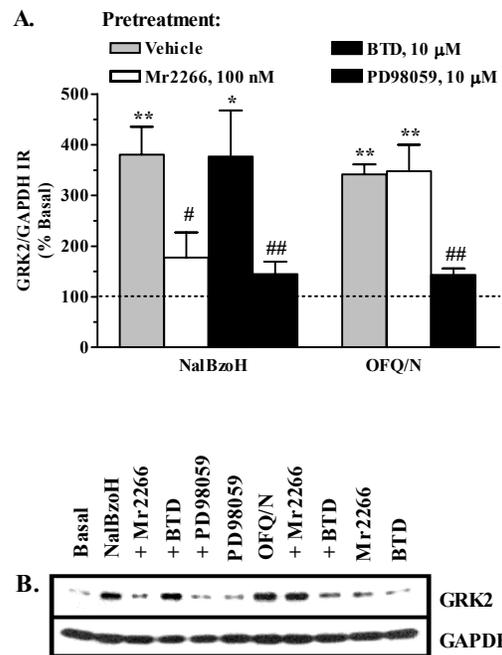


Fig. 7: Prolonged exposure to NalBzoH induces GRK2 levels via a receptor distinct from ORL1 via involvement of ERK1/2. Cells were pretreated with or without indicated concentrations of Mr2266 or BTD for 5 min or PD98059 for 60 min prior to stimulation with NalBzoH (1  $\mu$ M) or OFQ/N (0.1 nM) for 24 h as described in Methods. Cell lysates were immunoblotted with GRK2 and GAPDH antibodies. **A.** Densitometric analysis of immunoreactive bands is presented as the mean percentage increase  $\pm$  s.e.m. values over basal GRK2/GAPDH ratio ( $0.47 \pm 0.06$ ) from three to four separate experiments. \* $p < 0.05$ , \*\* $p < 0.01$ ; significantly different from basal and # $p < 0.05$ , ## $p < 0.01$ ; significantly different from drug-induced GRK2 levels. **B.** A representative immunoblot.

## DISCUSSION

In the present study, we provide further evidence suggesting that NalBzoH activates the  $\kappa_3$  opioid receptor and that it upregulates TH and GRK2 protein expression via stimulation of ERK1/2 through the  $\kappa_3$  opioid receptor. Several lines of evidence indicate that NalBzoH activates a receptor distinct from the NOP receptor to stimulate ERK1/2 activity in SH-SY5Y cells. Functionally, NalBzoH antagonizes the actions of  $\mu$  opioid receptor agonists<sup>[3-5,10]</sup> and, therefore is unlikely to activate ERK1/2 via the  $\mu$  opioid receptor in this cell line. In CHO cells expressing recombinant  $\mu$  opioid receptor, NalBzoH failed to activate ERK1/2 or inhibit cAMP (data not shown). We do not observe any significant ERK1/2 activation in SH-SY5Y cells following exposure to selective  $\mu$  (CTAP) or NOP (peptide III BTD) receptor antagonists at concentrations effective for blocking the actions of respective receptor agonists. These antagonists failed to inhibit NalBzoH-stimulated ERK1/2 activation (Fig. 2), suggesting that neither  $\mu$  nor NOP receptors were mediating the NalBzoH response in SH-SY5Y cells. Previous studies have clearly ruled out the involvement of the  $\delta$  opioid receptor, as its antagonists had no effect on the actions of NalBzoH<sup>[4, 10]</sup>. Mr2266, a  $\mu/\kappa$  receptor antagonist<sup>[35]</sup>, was previously found to inhibit  $\kappa_3$ , but not NOP, receptor signaling<sup>[10,12]</sup>. In the present study also, Mr2266 blocked the ERK1/2 phosphorylation resulting from NalBzoH, but not OFQ/N, exposure.

The conclusion that NalBzoH elicits its actions via a distinct receptor also is suggested by our observation that pretreatment with the NOP receptor agonist, OFQ/N, produced homologous desensitization of its subsequent ERK1/2 response but had no effect on NalBzoH-mediated ERK1/2 activation. Pretreatment with NalBzoH significantly diminished subsequent NalBzoH-stimulated ERK1/2 activation, confirming a previous report<sup>[12]</sup> that the  $\kappa_3$  receptor is subject to homologous desensitization. NalBzoH pretreatment also attenuated subsequent OFQ/N-mediated ERK1/2 stimulation in SH-SY5Y cells. To determine whether this one-way cross-tolerance was specific to ERK1/2 phosphorylation or could be generalized to other functions, we also examined the inhibition of forskolin-stimulated cAMP accumulation. As observed earlier, pretreatment with both OFQ/N and NalBzoH produced a homologous desensitization of their own cAMP inhibitory responses. NalBzoH pretreatment also desensitized NOP-mediated inhibition of forskolin-stimulated cAMP accumulation in SH-SY5Y cells. In those systems in which NalBzoH antagonized NOP receptor actions, it did so via competitive binding to the NOP receptor<sup>[6,18]</sup>. This is highly unlikely in our

studies as after NalBzoH pretreatment cells were extensively washed before OFQ/N application. Given the documented low affinity of NalBzoH for the NOP receptor, continued NalBzoH association with the receptor is unlikely. An increase in NalBzoH responsiveness following OFQ/N pretreatment was previously reported in BE(2)-C cells<sup>[12]</sup>. We also observed that OFQ/N pretreatment increases the potency of NalBzoH for inhibiting forskolin-stimulated cAMP accumulation in SH-SY5Y cells. Though suggestive, the increase in NalBzoH-mediated ERK1/2 activation following OFQ/N pretreatment did not reach statistical significance. The desensitization studies provide substantial evidence for distinguishing the actions of NalBzoH at a distinct receptor site from that activated by OFQ/N.

NalBzoH-mediated ERK1/2 activation is sensitive to PTX, suggesting the involvement of  $G_{i/o}$  proteins.  $G_{i/o}$  proteins are also involved in the analgesic action of NalBzoH<sup>[36]</sup> as well as in its inhibition of forskolin-stimulated cAMP accumulation in BE(2)-C cells<sup>[10]</sup>.  $G_i$ -mediated ERK signaling utilizes the  $\beta\gamma$  subunit of  $G_i$  and is PI3K, but not PKC-dependent, whereas  $G_o$ -mediated ERK activation involves the  $\alpha$  subunit of  $G_o$  along with PKC, but not PI3K<sup>[37]</sup>. NalBzoH-stimulated ERK1/2 activity was partially blocked by the PKC, but not the PI3K, inhibitor indicating the involvement of  $G_o$ , but not  $G_i$ , in this response. OFQ/N-mediated ERK1/2 signaling involves  $G_i$  and  $G_o$  proteins, and is partly mediated by PI3K as well as PKC as reported earlier<sup>[38]</sup>. It is possible that NalBzoH-mediated desensitization of ORL1 occurs as a result of PKC activation, since the NOP receptor is sensitive to PKC<sup>[39-41]</sup>.

In the present study, we observe that prolonged treatment with NalBzoH results in an ERK1/2-dependent induction of TH in SH-SY5Y cells, similar to that previously noted for  $\mu$  agonists<sup>[30,42,43]</sup>. This is in direct contrast to prolonged OFQ/N exposure that significantly reduces TH levels in SH-SY5Y cells<sup>[30]</sup>. NalBzoH-mediated upregulation of GRK2 levels in an ERK1/2-dependent fashion is similar to that seen with morphine and OFQ/N in SH-SY5Y cells<sup>[31]</sup>. The distinction, however, is that Mr2266, but not peptide III-BTD, blocked TH as well as GRK2 upregulation induced by NalBzoH, again indicating  $\kappa_3$ , and not NOP receptor-mediated effects. Moreover, treatment of cells for 24 hr with the  $\delta$ -selective opioid agonist DPDPE (1  $\mu$ M) failed to increase GRK2 and/or TH levels, indicating that NalBzoH's actions could not have been mediated through a  $\delta$  opioid receptor, and further support our hypothesis that NalBzoH's actions are mediated through a distinct  $\kappa_3$  opioid receptor.

In the present study, we illustrate for the first time that NalBzoH activates ERK1/2 through an opioid

receptor distinct from  $\mu$ ,  $\delta$ , and NOP, and demonstrate the potential significance of this signal in contributing to the chronic actions of NalBzoH. Although NalBzoH is not used clinically, other clinically useful agents such as levorphanol and nalbuphine also exhibit  $\kappa_3$  agonist properties<sup>[44,45]</sup> and may produce actions similar to NalBzoH with prolonged administration. We provide further evidence suggesting that NalBzoH elicits its agonist actions via a receptor distinct from that activated by OFQ/N, by demonstrating the differential sensitivity of OFQ/N and NalBzoH to receptor antagonists, kinase inhibitors and agonist pretreatments. However, in accordance with previous reports of NalBzoH interacting with the NOP receptor<sup>[6,18]</sup> as well as the  $\mu$  opioid receptor<sup>[3-7]</sup>, it is possible that NalBzoH activates a dimer resulting from association of the two receptors or their splice variants<sup>[46,47]</sup>. Indeed, NalBzoH affinity for the NOP receptor was increased in cell membranes containing the  $\mu$ -NOP receptor dimer<sup>[46]</sup>. Whether these dimers are functionally distinct from either receptor alone and are responsible for the actions of NalBzoH still remains to be elucidated. Nevertheless, the present study underscores the need to be cognizant of the many diverse actions of NalBzoH and other drugs with  $\kappa_3$  opioid agonist properties when utilizing these drugs in acute as well as long-term studies. Therefore, further examination of the  $\kappa_3$  versus NOP receptor properties of these agents and their cellular consequences is highly warranted.

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