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# The role of the ORL1 receptor in the modulation of spinal neurotransmission by nociceptin/orphanin FQ and nocistatin

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**Abstract**

Nociceptin/orphanin FQ and nocistatin are two neuropeptides with opposing effects on spinal neurotransmission and nociception. Nociceptin/orphanin FQ selectively suppresses excitatory glutamatergic neurotransmission, while nocistatin selectively interferes with glycinergic and γ-aminobutyric acid (GABA)-ergic transmission. Here, we performed whole-cell patch-clamp recordings from superficial rat spinal cord dorsal horn neurons to investigate the role of the opioid receptor-like (ORL)1 receptor for modulatory actions of these peptides. The partial ORL1 receptor antagonist [phe$^1\psi$($CH_2$–NH)Gly$^2$]nociceptin-(1–13)$NH_2$ competitively reversed the effects of nociceptin/orphanin FQ on excitatory neurotransmission (estimated p$A_2$ 6.43), but left the suppression of inhibitory synaptic transmission by nocistatin unaffected. These results indicate that the inhibitory action of nociceptin/orphanin FQ on glutamatergic transmission is mediated via ORL1 receptors, while nocistatin acts via a different so far unidentified receptor. © 2001 Published by Elsevier Science B.V.

*Keywords:* Nociceptin; Nocistatin; ORL1 receptor; Spinal cord, dorsal horn; Excitatory synaptic transmission; Inhibitory synaptic transmission; Nociception; Pain

## 1. Introduction

The neuropeptides nociceptin/orphanin FQ (Meunier et al., 1995; Reinscheid et al., 1995) and nocistatin (Okuda-Ashitaka et al., 1998) have repeatedly been implicated as modulators of several central nervous system (CNS) functions including nociception. Nociceptin/orphanin FQ, which is an endogenous agonist at the opioid receptor-like (ORL)1 receptor (e.g. Mollereau et al., 1994), exerts both pro- and anti-nociceptive effects probably depending on the dose and site of application (e.g. Meunier et al., 1995; Reinscheid et al., 1995; Erb et al., 1997; recently reviewed by Caló et al., 2000). Nocistatin, a second peptide derived from the same precursor peptide, antagonizes nociceptin/orphanin FQ- or prostaglandin $E_2$-induced allodynia and hyperalgesia (Okuda-Ashitaka et al., 1998). In certain pain models, it exhibits per se pro- or anti-nociceptive effects (Nakano et al., 2000; Zeilhofer et al., 2000).

In the spinal cord dorsal horn, which constitutes the first important site of synaptic integration in the pain pathway, both neurons expressing the precursor peptide pre-pro-nociceptin/orphanin FQ (Mollereau et al., 1996; Houtani et al., 1996) and ORL1 receptors, the presumed targets of nociceptin/orphanin FQ (Nishi et al., 1997; Narita et al., 1999), are present. L-Glutamate and glycine, together with γ-aminobutyric acid (GABA), serve as the major excitatory and inhibitory neurotransmitters in this CNS area. We and others have previously demonstrated that nociceptin/orphanin FQ selectively inhibits excitatory glutamatergic synaptic transmission in the spinal cord dorsal horn via a presynaptic mechanism (Liebel et al., 1997; Luo et al., 2000; Zeilhofer et al., 2000), while nocistatin only interferes with the release of the inhibitory neurotransmitters glycine and GABA (Zeilhofer et al., 2000).

Here, we show that the pseudopeptide [phe$^1\psi$($CH_2$–NH)Gly$^2$]nociceptin-(1–13)$NH_2$ (Guerrini et al., 1998), which is a partial ORL1 receptor antagonist (Bigoni et al., 1999; Connor et al., 1999; Okawa et al., 1999) with negligible affinity at classical (μ-, κ- and δ-) opioid recep-

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tors (Varani et al., 1999), competitively blocks the effects of nociceptin/orphanin FQ on excitatory postsynaptic currents (EPSCs), but does not interfere with the action of nocistatin on inhibitory postsynaptic currents (IPSCs).

## 2. Materials and methods

### 2.1. Slice preparation and electrophysiological recordings

Ten to 16-day-old Sprague–Dawley rats of either sex were killed in ether narcosis by decapitation. Two hundred fifty-micrometer-thick transverse slices of the lumbar spinal cord were prepared as described previously (Liebel et al., 1997). Whole-cell patch-clamp recordings were performed from neurons identified under visual control using the infrared gradient contrast technique coupled to a video microscopy system. Slices were completely submerged and continuously superfused with external solution, which contained (in mM) 125 NaCl, 26 $NaHCO_3$, 1.25 $NaH_2PO_4$, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose (pH 7.30, 315 mosMol/l), and was bubbled with 95% $O_2$, 5% $CO_2$. Patch pipettes (4–5 MΩ) were filled with internal solution containing (in mM) 130 K-gluconate, 20 KCl, 2 $MgCl_2$, 0.05 EGTA, 3 Na-ATP, 0.1 Na-GTP, 10 Na-HEPES (pH 7.30). Lidocaine *N*-ethyl bromide (QX-314) (5 mM) was added to the internal solution to block voltage-activated sodium currents and to improve space clamp conditions. EPSCs and IPSCs were evoked at a frequency of 0.1–0.07 Hz and recorded at −80 mV at room temperature. Short hyperpolarizing voltage steps to −90 mV were applied every minute to monitor input and access resistance. EPSCs and IPSCs were elicited by ipsilateral extracellular electrical stimulation (100 μs, 3–10 V) of the dorsal root entry zone using a glass electrode filled with 1 M NaCl. Peptide or drug-containing solutions were applied by bath perfusion at a rate of 1–2 ml/min. Percent inhibition of EPSCs and IPSCs by nociceptin/orphanin FQ and nocistatin was determined from the average amplitude of 10 consecutive PSCs evoked immediately before applica-

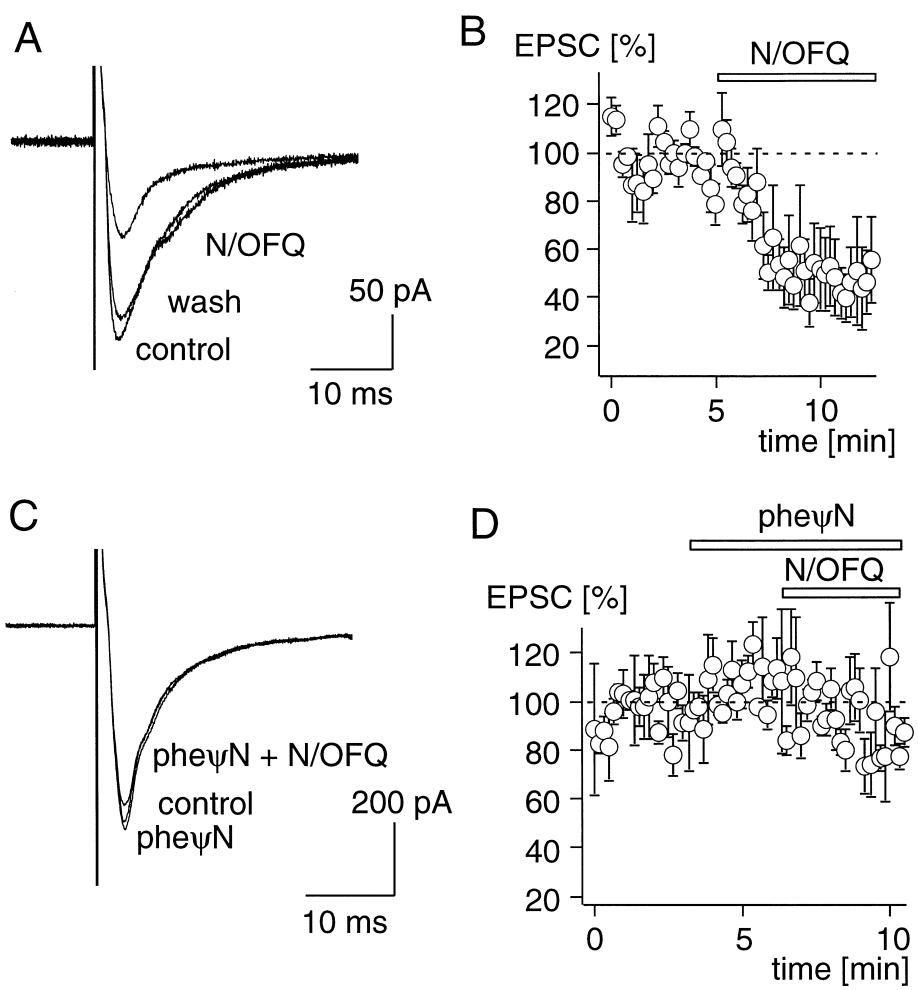


Fig. 1. [Phe$^1$ψ($CH_2$–NH)Gly$^2$]-nociceptin-(1–13)$NH_2$ antagonized the inhibitory effect of nociceptin/orphanin FQ on excitatory synaptic transmission. (A,C) Average current traces obtained from 10 consecutive EPSCs recorded at −80 mV under control conditions and in the presence of nociceptin/orphanin FQ (N/OFQ, 3 μM), [phe$^1$ψ($CH_2$–NH)Gly$^2$]nociceptin-(1–13)$NH_2$ (pheψN, 10 μM) or both. (B,D) Average EPSC amplitudes (mean ± S.E.M.) plotted versus time ($n = 8$ and 6, in (B) and (D), respectively).

tion of the peptides and when a steady state of inhibition was reached, usually about 3 min after peptide application. The killing of the animals was performed in accordance with the institutional guidelines of the University of Erlangen-Nürnberg and of the European Community.

### 2.2. Peptides

Bovine nocistatin was purchased from Tocris (Bristol, UK). Nociceptin/orphanin FQ was obtained from Dr. M. Herkert (Institut für Biochemie, Universität Erlangen-Nürnberg, Germany) and from Tocris. [Phe$^1\psi$(CH$_2$–NH)Gly$^2$]nociceptin-(1–13)NH$_2$ was obtained from Sigma (Deisenhofen, Germany). Peptides (purity > 95%) were dissolved in external recording solution and stored in aliquots (1 mM) at −20°C. Fresh dilutions were made with standard external solution on every experimental day.

## 3. Results

### 3.1. [Phe$^1\psi$(CH$_2$–NH)Gly$^2$]nociceptin-(1–13)NH$_2$ competitively antagonizes nociceptin / orphanin FQ-mediated inhibition of EPSCs

Excitatory synaptic transmission was studied in the presence of strychnine (2 μM) and bicuculline (10 μM). EPSCs could almost completely be blocked by a combination of the non-*N*-methyl-D-aspartate (non-NMDA) receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and the NMDA receptor antagonist D-(−)-2-amino-5-phosphonovaleric acid (D-APV, 50 μM), indicating that they were mediated by ionotropic glutamate receptors.

Application of nociceptin/orphanin FQ (3 μM) led to a reversible reduction of the EPSC amplitudes by 42.5 ± 12.3% (mean ± S.E.M.; $n = 5$; Fig. 1A,B). This inhibitory effect was significantly reduced to 11.1 ± 4.61 % ($n = 6$) by the partial nociceptin/orphanin FQ receptor antagonist [phe$^1\psi$(CH$_2$–NH)Gly$^2$]nociceptin-(1–13)NH$_2$ (10 μM; Fig. 1C). At this concentration, [phe$^1\psi$(CH$_2$–NH)Gly$^2$]-nociceptin-(1–13)NH$_2$ did not inhibit EPSC amplitudes by itself. On average, EPSC amplitudes even increased slightly by 8.4 ± 1.6 % ($n = 9$; Fig. 1D).

Fig. 2A and B show the concentration–response curves of nociceptin/orphanin FQ-mediated reduction of EPSC amplitudes. Under control conditions, nociceptin/orphanin FQ reduced EPSC amplitudes by 43.2 ± 6.9% at saturating concentrations and with an EC$_{50}$ of 496 ± 224 nM. [Phe$^1\psi$(CH$_2$–NH)Gly$^2$]nociceptin-(1–13)NH$_2$ (10 μM) shifted the concentration–response curve to the right giving rise to a new EC$_{50}$ of 9.3 μM, while maximum EPSC reduction and the Hill coefficient remained almost constant ($E_{max}$: 43.0% versus 43.2%; $n_H$: 0.94 versus 1.01, in the presence and absence of [phe$^1\psi$(CH$_2$–NH)Gly$^2$]nociceptin-(1–13)NH$_2$, respectively). These findings suggest a competitive antagonism between nociceptin/orphanin FQ and [phe$^1\psi$(CH$_2$–NH)Gly$^2$]nociceptin-(1–13)NH$_2$. Reversal of nociceptin/orphanin FQ-mediated reduction of spinal EPSCs by [phe$^1\psi$(CH$_2$–NH)Gly$^2$]nociceptin-(1–13)NH$_2$ occurred in a concentration-dependent manner (Fig. 2B). A p$A_2$ of 6.43 was estimated from Schild analysis. At the highest concentration tested (100 μM), [phe$^1\psi$(CH$_2$–NH)Gly$^2$]nociceptin-(1–13)NH$_2$ reduced the amplitudes

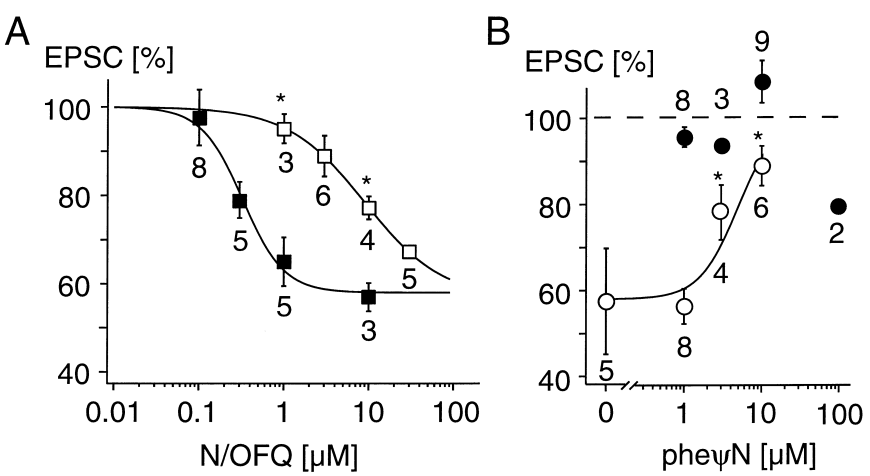


Fig. 2. (A) Concentration–response curves of nociceptin/orphanin FQ in the absence (■) and presence (□) of [phe$^1\psi$(CH$_2$–NH)Gly$^2$]nociceptin-(1–13)NH$_2$ (10 μM). $^*p \leq = 0.05$ indicate statistically significant reduction of nociceptin/orphanin FQ-mediated inhibition by [phe$^1\psi$(CH$_2$–NH)Gly$^2$]nociceptin-(1–13)NH$_2$ (Welch test). (B) Concentration–response curves of [phe$^1\psi$(CH$_2$–NH)Gly$^2$]nociceptin-(1–13)NH$_2$ alone (●) and in the presence of nociceptin/orphanin FQ (3 μM; ○). Data points were fitted to the equation $y = y_{max} - [(y_{max} - y_{min})/(1 + (EC_{50}/C)_{nH})]$ using the Jandel Sigma Plot 3.0 software (Jandel, Erkrath, Germany). $y_{max}$ is the normalized EPSC amplitude under control conditions, $y_{min}$ the relative EPSC amplitude in the presence of saturating concentrations of nociceptin/orphanin FQ, $C$ is the nociceptin/orphanin FQ concentration, EC$_{50}$ is the half maximum effective concentration of nociceptin/orphanin FQ and $n_H$ is the Hill coefficient. $^*p \leq 0.05$ indicates statistically significant reduction of nociceptin/orphanin FQ-mediated inhibition by [phe$^1\psi$(CH$_2$–NH)Gly$^2$]nociceptin-(1–13)NH$_2$ (ANOVA, followed by Bonferroni post hoc test). Numbers below and above data points are the number of cells.

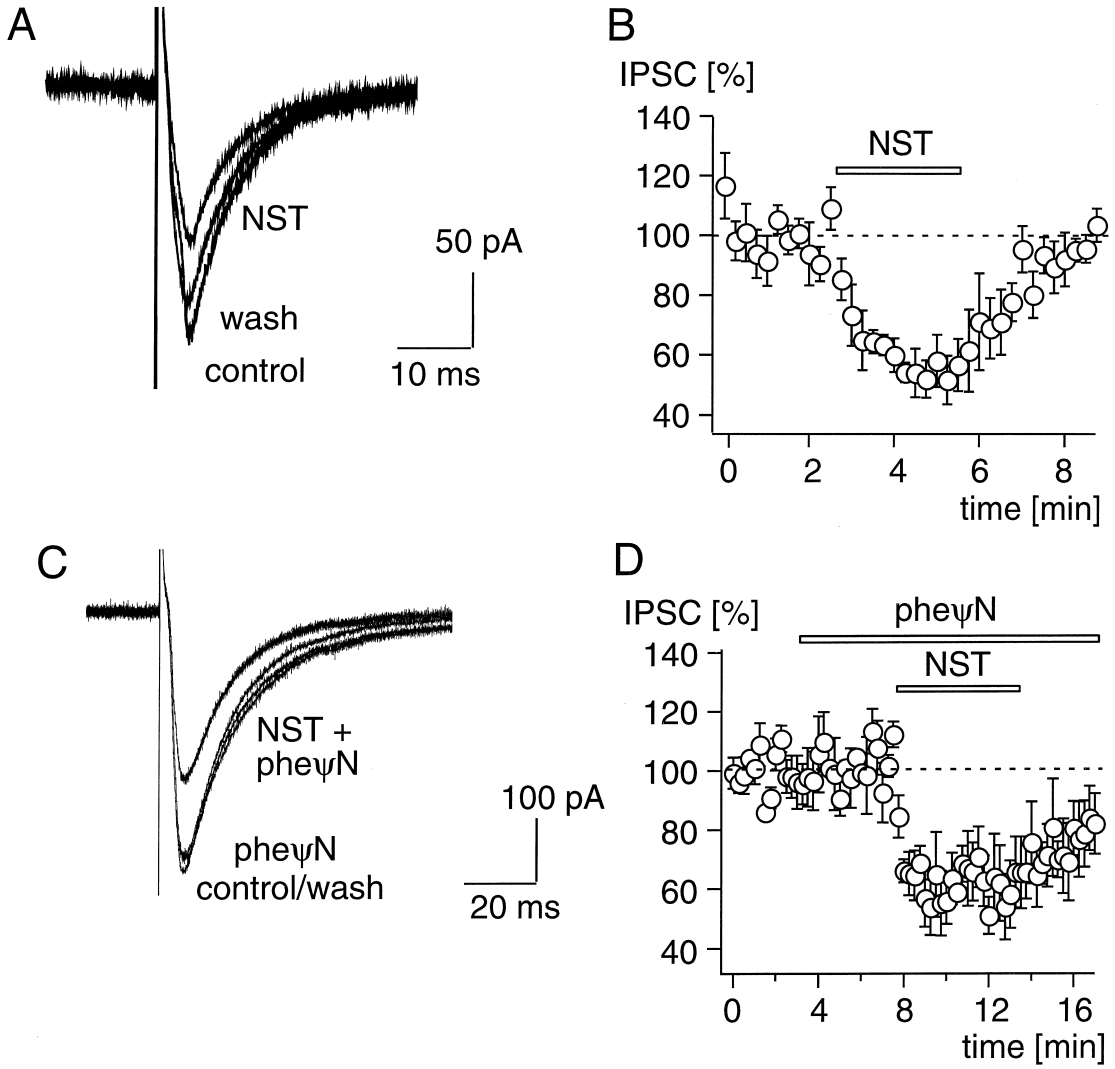


Fig. 3. Effects of [phe$^1\psi$($CH_2$–NH)Gly$^2$]nociceptin-(1–13)$NH_2$ on nocistatin (NST)-mediated inhibition of IPSCs. (A,B) Average current traces obtained from 10 consecutive IPSCs recorded at −80 mV under control conditions and in the presence of nocistatin (3 μM) or/and [phe$^1\psi$($CH_2$–NH)Gly$^2$]nociceptin-(1–13)$NH_2$ (pheψN, 10 μM). (C,D) Average IPSC amplitudes (mean ± S.E.M.) plotted versus time ($n = 6$ and 5, in (B) and (D), respectively).

of EPSCs suggesting weak agonistic activity at ORL1 receptors in the rat spinal cord.

### 3.2. [Phe$^1\psi$($CH_2$–NH)Gly$^2$]nociceptin-(1–13)$NH_2$ does not affect inhibition of IPCs by nocistatin

In a separate set of experiments, the effect of [phe$^1\psi$($CH_2$–NH)Gly$^2$]nociceptin-(1–13)$NH_2$ on nocistatin-mediated reduction of inhibitory synaptic transmission was investigated. IPSCs, which were recorded in isolation using the glutamate receptor antagonists CNQX (10 μM) and D-APV (50 μM), were almost completely blocked by a combination of bicuculline (10 μM) and strychnine (2 μM).

At a saturating concentration of 3 μM (Zeilhofer et al., 2000), nocistatin reduced the amplitudes of IPSCs by 39.6 ± 3.7% ($p = 0.05$; $n = 5$; ANOVA; Fig. 3A). The degree of inhibition was almost identical (35.2 ± 5.6%; $n = 5$; Fig. 3B) in the presence of [phe$^1\psi$($CH_2$–NH)Gly$^2$]nociceptin-(1–13)$NH_2$ (10 μM). [Phe$^1\psi$($CH_2$–NH)Gly$^2$]nociceptin-(1–13)$NH_2$ itself did not interfere with IPSCs. The change in IPSC amplitudes was less than 0.8 ± 0.045% ($n = 5$).

## 4. Discussion

In the present study, we have shown that the inhibitory effect of nociceptin/orphanin FQ on excitatory synaptic transmission is antagonized by [phe$^1\psi$($CH_2$–NH)Gly$^2$] nociceptin-(1–13)$NH_2$, while that of nocistatin on inhibitory synaptic transmission is not. These findings clearly indicate that nociceptin/orphanin FQ acts via ORL1 receptors, while the action of nocistatin is independent of this receptor.

Reduction by nociceptin/orphanin FQ of EPSC amplitudes in the rat spinal cord dorsal horn occurs at relatively high concentrations ($EC_{50} \approx 500$ nM; Liebel et al., 1997, and this study; $EC_{50} \approx 200$ nM Luo et al., 2000). Although nociceptin/orphanin FQ is more potent in other preparations (e.g. Meis and Pape, 1998; Connor et al., 1999), the sensitivity of this inhibition to [phe$^1\psi$($CH_2$–NH)Gly$^2$] nociceptin-(1–13)$NH_2$ indicates that it is nevertheless mediated via ORL1 receptors. In addition, our finding that the estimated p$A_2$ value for [phe$^1\psi$($CH_2$–NH)Gly$^2$]nociceptin-(1–13)$NH_2$ (6.43) in the rat spinal cord is similar to those reported for other central and peripheral preparations (reviewed by Caló et al., 2000) argues against receptor heterogeneity as a reason for the different potencies. An alternative and more likely explanation is that differences in ORL1 receptor density and receptor reserve in different preparations strongly affect the potency of nociceptin/orphanin FQ.

[Phe$^1\psi$($CH_2$–NH)Gly$^2$]nociceptin-(1–13)$NH_2$ has convincingly been demonstrated to exert antagonistic and agonistic activity both in vitro (e.g. Butour et al., 1998; Meis and Pape, 1998) and in vivo (e.g. Carpenter and Dickenson, 1998; Armstead, 1999; Wang et al., 1999). Which of these actions dominates may depend on the density of ORL1 receptors and down stream transducer molecules, e.g. G-proteins. In case of a small receptor reserve, the low intrinsic activity of [phe$^1\psi$($CH_2$–NH)Gly$^2$]nociceptin-(1–13)$NH_2$ (Bigoni et al., 1999; Okawa et al., 1999) will favor antagonistic actions, while agonistic effects may dominate in tissues with a high receptor reserve (see also Toll et al., 1998; Caló et al., 2000). Both ORL1 receptor expression and their coupling to G-proteins may, in addition, be developmentally regulated. The latter possibility might explain why antagonistic actions have been frequently reported in CNS or spinal cord slices, which are in most cases taken from newborn or young animals, while studies performed in intact usually adult animals mainly report agonistic effects.

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