Roles of nociceptin/orphanin FQ and nociceptin/orphanin FQ peptide receptor in respiratory rhythm generation in the medulla oblongata: an in vitro study

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Background. Nociceptin/orphanin FQ (N/OFQ) is the endogenous agonist of the orphan opioid receptor-like receptor (NOP receptor, previously termed ORL1), a novel member of the opioid receptor family. The aim of the present study, using in vitro newborn rat preparations, was to elucidate the roles N/OFQ and the NOP receptor play in medullary generation of respiratory rhythm.

Methods. The brainstem-spinal cord from 3-day-old Wistar rats was isolated and perfused with artificial cerebrospinal fluid (27.5°C) equilibrated with oxygen 95% and carbon dioxide 5% at pH 7.4. Respiratory activity was recorded from the C4/C5 ventral roots. The effects of N/OFQ (10 nM, 30 nM, 100 nM) on respiratory frequency (fR) (bursts min⁻¹) was measured. Drugs were administered through the recording chamber by means of a perfusion system. In addition, the effects of pretreatment with the classical non-selective opioid receptor antagonist naloxone 1 μM, and the selective NOP antagonist CompB 10 μM, were evaluated. Statistical significance was evaluated using ANOVA followed by Dunnett’s test (P<0.05).

Results. N/OFQ reduced fR in a concentration-dependent manner. Pretreatment with CompB 10 μM prevented the N/OFQ 10 nM-induced fR reduction, whereas CompB itself was inactive. Pretreatment with naloxone did not prevent the N/OFQ-induced fR reduction.

Conclusion. N/OFQ acts as a neuromodulator to reduce fR in the respiratory rhythm-generating centre of the medulla oblongata, and this action of N/OFQ is mediated by NOP receptors.

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In addition to the three classical opioid receptors (μ, δ, and κ) a new member of the opioid receptor family, nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor (previously termed ORL1 receptor), was cloned in 1994.1,2 Although structurally related to other members of the opioid receptor family, this receptor was found to bind to classical μ-, δ-, and κ-opioid ligands with very low affinity. In 1995, N/OFQ was isolated and identified as the endogenous ligand for the NOP receptor.3,4 NOP receptor and N/OFQ are involved in many physiological functions.5 Whilst abundance of the NOP receptor in the medulla oblongata has been noted,6,7 the roles of the NOP receptor and N/OFQ specifically in the respiratory centre of the medulla oblongata have not yet been described.

In vitro brainstem-spinal cord preparation from newborn rat has been introduced as an in vitro model for the study of the mammalian respiratory centre.8 Cumulative evidence has shown that this model is well suited to the analysis of primary respiratory rhythm generation and modulation.8-10 A considerable number of studies examining the effects of neuroactive substances on the respiratory centre have been conducted using this in vitro preparation10 for which there are several advantages. Most notably, because this preparation can be maintained in an anaesthetic-free condition, the
direct effects of neuroactive substances and drugs on the structures that generate basal respiratory rhythm can be observed. In addition, this environment precludes the influence of peripheral chemosensors and suprabulbar structures.

The aim of the present study was, using the above model, to elucidate the roles of N/OFQ and the NOP receptor in respiratory rhythm generation in the medulla oblongata.

**Methods**

This study was approved by the Animal Care Committee of the Hokkaido University Graduate School of Medicine. The head and upper thorax of Wistar rats (3 days old, n=48) were dissected under deep ether anaesthesia, and the brainstem and spinal cord were isolated. The brainstem was rostrally decerebrated between the VIth cranial nerve roots and the lower border of the trapezoid body. The preparation was placed in a small chamber (volume 2.5 ml) and continuously perfused at a rate of 5.0±6.0 ml min⁻¹ with artificial cerebrospinal fluid (aCSF) (mM): NaCl, 124; KCl, 5.0; KH₂PO₄, 1.2; CaCl₂, 2.4; MgSO₄, 1.3; NaHCO₃, 26; glucose, 30. The solution was equilibrated with oxygen 95% and carbon dioxide 5% to pH 7.4 at 27.5°C. The reservoir containing the aCSF, the perfusion system, and the recording chamber was heated using a water bath to 27.5 (SD 0.3)°C. The pH and temperature of the aCSF in the reservoir, and the temperature of the superfusate in the recording chamber, were continuously monitored.

Respiratory activity corresponding to inspiration was monitored at the C4 or C5 ventral root using suction electrodes; this activity is known to synchronize with phrenic nerve discharges and with contraction of the inspiratory intercostal muscles. Recording signals were amplified and band-pass filtered (50 Hz to 3 kHz; Fig. 1).

More than 20 min after the recording of respiratory activity was established, drugs were administered through the recording chamber via the perfusion system. N/OFQ 10 nM, 30 nM, or 100 nM was applied for 20 min with each preparation being exposed once to a single concentration of N/OFQ. In the five preparations used as non-treated controls, no drug was applied. Naloxone 1 μM, the putative NOP antagonist 1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxy-methyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one (CompB) 10 μM or [Phe¹ψ(CH₂-NH)Gly²]-N/OFQ (1–13)-NH₂ 1–3 μM were applied 20 min before co-application with N/OFQ 10 nM for a further 20 min. In some experiments, after 20 min exposure to N/OFQ 10 nM, the combination of N/OFQ and CompB 10 μM was applied. N/OFQ, naloxone hydrochloride, and [Phe¹ψ(CH₂-NH)Gly²]-

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**Fig 1** Schematic diagram of the newborn rat brainstem-spinal cord preparation ventral surface. Respiratory motoneuron activity corresponding to inspiration was recorded from the C4/C5 ventral root through a glass suction electrode. AICA=anterior inferior cerebellar artery; IX-XII=cranial nerves; and C1–C4=cervical ventral nerves.
nociceptin (1–13)-NH₂ were obtained from Sigma (St Louis, MO, USA). CompB was a generous gift from Banyu Pharmaceutical Company (Tokyo, Japan).

Respiratory frequency (fR) was measured with firing frequency of C4 activity regarded as fR. Average values were calculated from the number of bursts recorded over a 3 min period. All data are presented as mean (SD). Statistical significance was evaluated using the Student’s t-test and analysis of variance (ANOVA) followed by Dunnett’s test. A value of P<0.05 was considered statistically significant.

### Results

N/OFQ reduced fR in a concentration-dependent manner (Table 1). The effect of N/OFQ lasted 30–60 min after peptide removal. In the preparation in which the dorsal half of the brainstem was removed (n=4), N/OFQ 10 nM also caused an fR reduction [8.3 (2.5) bursts min⁻¹ to 4.3 (3.0) bursts min⁻¹, P<0.05]. The effects of pretreatment with naloxone, CompB and [Phe¹Ψ(CH₂-NH)Gly²]-N/OFQ (1–13)-NH₂ on the actions of N/OFQ are shown in Table 2. Naloxone 1 µM pretreatment did not prevent N/OFQ 10 nM-induced fR reduction. CompB 10 µM pretreatment prevented N/OFQ 10 nM-induced fR reduction, and had no significant effects on fR alone. CompB 10 µM also reversed N/OFQ 10 nM-induced fR reduction [6.6 (2.7) bursts min⁻¹ to 9.8 (2.3) bursts min⁻¹, P<0.05, n=5; Fig. 2]. [Phe¹Ψ(CH₂-NH)Gly²]-N/OFQ (1–13)-NH₂ 1 µM pretreatment did not prevent N/OFQ 10 nM-induced fR reduction, but higher concentrations of [Phe¹Ψ(CH₂-NH)Gly²]-N/OFQ (1–13)-NH₂, 3 µM caused fR reduction per se.

### Discussion

The current study demonstrated that N/OFQ has an inhibitory effect on the medullary structures involved in

### Table 1

<table>
<thead>
<tr>
<th>Concentration (nM)</th>
<th>0 (control)</th>
<th>10</th>
<th>30</th>
<th>100</th>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>fR (bursts min⁻¹)</td>
<td>Baseline</td>
<td>9.7 (1.6)</td>
<td>10.3 (2.8)</td>
<td>10.0 (1.5)</td>
</tr>
<tr>
<td>20 min after N/OFQ</td>
<td>9.4 (0.5)</td>
<td>5.6 (0.5)²</td>
<td>3.5 (1.9)²</td>
<td>1.0 (0.4)²</td>
</tr>
<tr>
<td>% of baseline value</td>
<td>98.3 (12.2)</td>
<td>59.3 (16.3)²</td>
<td>36.5 (23.3)²</td>
<td>8.4 (2.5)²</td>
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</table>

### Table 2

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>n</th>
<th>fR (bursts min⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>Naloxone</td>
<td></td>
<td>Baseline</td>
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<tr>
<td>1 µM</td>
<td>5</td>
<td>8.7 (2.1)</td>
</tr>
<tr>
<td>CompB</td>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>10 µM</td>
<td>6</td>
<td>10.9 (0.8)</td>
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<tr>
<td>Phe¹Ψ</td>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>1 µM</td>
<td>4</td>
<td>10.5 (2.1)</td>
</tr>
<tr>
<td>3 µM</td>
<td>4</td>
<td>8.4 (1.7)</td>
</tr>
<tr>
<td>N/OFQ 10 nM</td>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>1 µM</td>
<td>5</td>
<td>8.4 (2.7)</td>
</tr>
<tr>
<td>CompB 10 µM</td>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>1 µM</td>
<td>5</td>
<td>4.3 (1.5)²</td>
</tr>
<tr>
<td>Phe¹Ψ 3 µM</td>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>4</td>
<td>5.5 (2.9)</td>
<td></td>
</tr>
<tr>
<td>N/OFQ 10 nM+</td>
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<td>Baseline</td>
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<tr>
<td>1 µM</td>
<td>5</td>
<td>10.3 (1.7)</td>
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<tr>
<td>CompB 10 µM</td>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>1 µM</td>
<td>5</td>
<td>5.5 (2.9)</td>
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</tbody>
</table>

Fig 2 (A) C4 activity recorded from the in vitro preparation in standard solution. (b) 20 min after perfusion with nociceptin/orphanin FQ 10 nM. (c) 15 min after perfusion with nociceptin/orphanin FQ 10 nM and CompB 10 µM.
the generation of respiratory rhythm via activation of the NOP receptor, a new member of the opioid receptor family.

Since N/OFQ was discovered in 1995, many studies have been performed to elucidate its actions on in vivo activity when injected intracerebroventricularly or microinjected into restricted areas of the central nervous system, of animals including rats and mice. Those studies showed that N/OFQ has many physiological and pharmacological actions in vivo, including nociception; analgesia; effects on locomotion, learning, cardiovascular control and inhibition of cough. However, there has been no report of respiratory effects of N/OFQ. This discrepancy between the results of the present in vitro study and those of previous in vivo studies suggests that the effects of N/OFQ on suprabulbar structures may modify medullary NOP receptor-mediated respiratory depressant effect.

CompB is reported to be a potent and selective NOP receptor antagonist. The results of the current series of experiments using CompB show that N/OFQ in the medullary structures is able to modulate respiratory rhythm and this effect is mediated by NOP receptors. The present study also showed that CompB had no effect on fR generation per se indicating that endogenous N/OFQ does not participate in basal respiratory rhythm generation in the medulla oblongata. [Phe1-ψ(CH2-NH)Gly2]-N/OFQ (1–13)–NH2 was introduced as a competitive antagonist of the NOP receptor in guinea-pig ileum and mouse vas deferens preparations. However, other studies have shown that this peptide acts as an agonist or a partial agonist for the NOP receptor in vitro and in vivo, although the reason for the differences in its pharmacodynamic profile is unclear. The diversity of this agent’s actions on the NOP receptor limits its utility as a pharmacological tool for manipulating the NOP receptor. The present study showed that [Phe1-ψ(CH2-NH)Gly2]-N/OFQ (1–13)–NH2 acted mainly as an agonist for the NOP receptor. In the present study, pretreatment with naloxone did not affect the N/OFQ-induced fR reduction, whereas findings in our previous study showed that naloxone can completely reverse the fR reduction produced by naloxone.

These findings suggest that N/OFQ-induced fR reduction observed here may result from activation of these NOP receptors.

We conclude that N/OFQ acts as a neuromodulator to reduce respiratory frequency in the respiratory rhythm-generation centre of the medulla oblongata, and that this action of N/OFQ is mediated by NOP receptors in the ventral portion of this structure.

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