

## Molecular characterization and functional expression of opioid receptor-like<sub>1</sub> receptor<sup>1</sup>

WU YA LAN, LU PU, KUN LING, JIAN ZHAO, ZHI JIE CHENG, LAN MA\*, GANG PEI<sup>2</sup>

*Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai 200031, China*

*\* Shanghai Medical University, Shanghai 200032, China*

### ABSTRACT

The opioid receptor-like<sub>1</sub> receptor (ORL<sub>1</sub>), an orphan receptor whose human and murine complementary DNAs, has been characterized recently. ORL<sub>1</sub> transcripts are particularly abundant in the central nervous system. We demonstrated that ORL<sub>1</sub> expressed in human neuroblastoma SK-N-SH and SH-SY5Y cell lines by radioligand binding assay, reverse transcription polymerase chain reaction (RT-PCR) and Northern analysis in the present study. Stimulation with ORL<sub>1</sub> specific agonist, nociceptin/orphanin FQ, increased [<sup>35</sup>S]GTP γ S binding to SK-N-SH cell membranes (EC<sub>50</sub> = 14 ± 0.45 nM), and attenuated, forskolin-stimulated accumulation of cellular cAMP (EC<sub>50</sub> = 0.80 ± 0.45 nM), indicative that activation of ORL<sub>1</sub> activates G proteins and inhibits adenylyl cyclase. Activation of ORL<sub>1</sub> receptor was also accessed using CHO:hORL<sub>1</sub> cell line by microphysiometer. Treatment of nociceptin/orphanin FQ increased extracellular acidification rate significantly.

**Key words:** *Opioid receptor-like<sub>1</sub> receptor (ORL<sub>1</sub>), nociceptin/orphanin FQ (N/OFQ), SK-N-SH cells, extracellular acidification, functional expression.*

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1. Dedicated to Professor Lu Ji SHI for his 80th birthday

2. To whom correspondence should be addressed.

## INTRODUCTION

Opioid acts in the central and peripheral nervous system (CNS and PNS) to produce numerous pharmacological effects. Repetitive or continuous use of opioids, however, causes drug tolerance and dependence. Three subtypes of opioid receptors, termed  $\mu$ ,  $\delta$ , and  $\kappa$ , have been cloned. They are coupled to the inhibitory G protein and negatively regulate adenylyl cyclase[1]. Recently a new G-protein-coupled receptor termed opioid receptor-like receptor (ORL<sub>1</sub>) which belongs to the newly characterized opioid receptor subfamily had been cloned from human brainstem cDNA libraries[2-8]. Its endogenous specific agonist N/OFQ has also been identified[9, 10], and it expresses mainly in the CNS[1, 4, 6, 7]. In spite of the fact that ORL<sub>1</sub> shares over 50 % homology with opioid receptors, it has very low affinity for the opioid ligands[5]. N/OFQ treatment causes inhibition of forskolin-stimulated cAMP accumulation in Chinese hamster ovary (CHO) cells exogenously transfected with ORL<sub>1</sub> cDNA[9, 10], stimulation of [<sup>35</sup>S]GTP- $\gamma$ S binding in rat brain[11] and increase of inwardly rectifying K<sup>+</sup> conductance in rat dorsal raphe nucleus neurons[12]. However, the information on signal transduction mediated by ORL<sub>1</sub> in a neuronal cellular system is lacking. In the present study, we have demonstrated that both human neuroblastoma SK-N-SH cells, which express the  $\mu$  and  $\delta$  opioid receptors[13], and SH-SY5Y cells also endogenously express ORL<sub>1</sub>, and that stimulation of SK-N-SH cells with N/OFQ, a specific ligand of ORL<sub>1</sub>, leads to activation of inhibitory G-proteins.

## MATERIALS AND METHODS

### *Cell lines and cell culture*

Human neuroblastoma SK-N-SH cells (American Type Culture Collection) were cultured in RPMI 1640 (Gibco-BRL) supplemented with 10% heat-inactivated fetal calf serum (Evergreen, Hangzhou, China). Chinese Hamster Ovary (CHO) cells and CHO:hORL<sub>1</sub> cells were cultured in DMEM (Gibco-BRL) containing 10% heat-inactivated calf serum (Evergreen). Human neuroblastoma SH-SY5Y cells were cultured in MEM (Gibco-BRL) containing 15% heat-inactivated fetal calf serum (Gibco-BRL). The media were also supplemented with 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin.

### *Isolation of total RNA*

$2 \times 10^6$  cultured cells were collected by centrifugation and lysed in 1 ml of TRIzol Reagent (Gibco-BRL) by repetitive pipetting. Total RNA was extracted according to the manufacturer-provided protocols from cultured cells and dissolved in diethylpyrocarbonate (DEPC) treated water.

### *Reverse transcription polymerase chain reaction (RT-PCR)*

First-strand cDNA was made from total RNA by using Superscript preamplification system (Gibco-BRL) and following the procedures suggested by the manufacturer. 2  $\mu$ g of total RNA was heated to 70°C for 10 min in 11  $\mu$ l of DEPC-treated water containing 0.5  $\mu$ g of oligo(dT)<sub>12-18</sub>. 4  $\mu$ l of 5  $\times$  synthesis Buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl<sub>2</sub>], 1  $\mu$ l of 10 mM dNTP mix, 2  $\mu$ l of 0.1 M DTT, 1  $\mu$ l of RNasin (20U/ $\mu$ l) and 1  $\mu$ l of reverse transcriptase (SuperScript II RT; 200U/ $\mu$ l) were added to the sample. The reaction mixture was incubated at

room temperature for 10 min and subsequently at 42 °C for 50 min. The reaction was terminated by incubating the mixture at 70 °C for 10 min. After cooling on ice for 10 min, 1  $\mu$ l of RNase H (2U/ $\mu$ l) was added to the mixture and then incubated for 20 min at 37 °C.

PCR primers used were ORL<sub>1</sub>FBamH and ORL<sub>1</sub>REcoR. These two primers correspond to regions from bases 171 to 188 and 1293 to 1310 on human ORL<sub>1</sub> cDNA[2] respectively. The other pair of primers, ORT5 (GGCACGGACATCCTCCTGGGCTTC) and ORT3 (GGGATCTC-CACCAGGCACTCGATC) correspond to regions from bases 497 to 515 and 766 to 784 respectively. The PCR reactions (50  $\mu$ l) contained 5  $\mu$ l of first-strand cDNA, 2 units of Taq polymerase (Promega). The conditions used were: 94 °C 1 min; 60 °C for 1 min; 72 °C for 1.5 min. This cycle was repeated 30 times. The PCR products (1150 bp and 300 bp respectively) were analyzed on a 1% agarose gel.

### *Cloning of human ORL<sub>1</sub> cDNA by polymerase chain reaction*

PCR primers used were ORL<sub>1</sub>FBamH (CGCGGATCCAGTGGCATGGAGCCC CTCTTCCC) and ORL<sub>1</sub>REcoR (CAGGAATTCATGGGCAGGTCCACGCCTAGTC). These primers are designed according to the sequence reported previously[2] with the restriction sites underlined and synthesized by GENOSYS. The reaction (50  $\mu$ l) contained 10 ng of human Brain cDNA (Clontech Laboratories), 2 units of Taq polymerase (Promega). The PCR conditions were just like above. PCR products were purified from a 1% agarose gel using QIAEX Gel Extraction Kit, digested with BamH I and EcoR I, and subcloned into pcDNA<sub>3</sub> (Invitrogen). This BamH I and EcoR I fragment containing the entire coding region of human ORL<sub>1</sub>. Nucleotide sequence analysis was performed using the Sequenase 2.0 DNA sequencing kit (Amersham) according to the manufacturer-suggested protocol.

### *Northern blot analysis*

Total RNA of each sample was electrophoresed on a 1% agarose-glyoxal gel (20  $\mu$ g/lane), transferred onto a nylon membrane (Amersham) with 20  $\times$  SSC (1  $\times$  SSC is 0.15 M Sodium Chloride, 0.15 M Sodium Citrate, pH 7.0). Then the membrane was exposed to UV light using a GS Gene Linker (Bio-Rad).

Gene-specific probe was prepared from cloned pcDNA<sub>3</sub>:hORL<sub>1</sub>. The plasmid was digested with BamH I and EcoR I. The 1150 bp restriction fragment containing entire coding region of ORL<sub>1</sub> was purified using QIAEX Gel Extraction Kit (QIAEX). <sup>32</sup>P-labeled random-primed probe was made to a specific activity of 5  $\times$  10<sup>8</sup> dpm/ $\mu$ g DNA using the Ready To Go DNA Labeling Kit (Pharmacia Biotech).

The membrane was perhybridized in 0.5 M phosphate buffer (pH 7.2), 7% SDS (Sodium Dodecyl Sulfate), 1 mM EDTA for 2 h at 65 °C, and hybridized to <sup>32</sup>P-labeled probe for 20 h at 65 °C [14]. The membrane was washed twice in 2  $\times$  SSC/0.1% SDS at room temperature for 15 min each, and then twice in 0.5  $\times$  SSC/0.1% SDS at 55 °C for 15 min each. The membrane was exposed to X-ray film (Kodak) for 24 - 72 h.

### *Construction of CHO:hORL<sub>1</sub> stable cell line*

CHO cells grown in 60 mm dishes were transfected with 10  $\mu$ g/dish of pcDNA3:hORL<sub>1</sub> by calcium phosphate precipitation[15]. 24 h after transfection, the cells were treated with trypsin and diluted 1:10. The following day, selection of transfectants was initiated by addition of 1 mg/ml Geneticin (Gibco-BRL). Two weeks later, individual resistant clones were isolated, cultured separately and checked for the presence of hORL<sub>1</sub> transcripts by using saturation binding[16] with [<sup>3</sup>H]-N/OEQ.

### *[<sup>35</sup>S]GTP $\gamma$ S binding assay*

The experiments were performed as described previously[11]. Cells were lysed in buffer contain-

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ing 5 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 5 mM EGTA and the cell lysate was centrifuged at  $30,000 \times g$  for 10 min. The membranes (containing 15  $\mu\text{g}$  protein) were incubated in 50 mM Tris-HCl, pH 7.5, 5 mM  $\text{MgCl}_2$ , 1 mM EGTA, 100 mM NaCl, 40  $\mu\text{M}$  GDP, and 8 nM [<sup>35</sup>S]GTP  $\gamma$  S (1200 Ci/mmol, DuPont-New England Nuclear), in a total volume of 0.1 ml. The incubation was started by adding the membrane suspension to the reaction mixture and was carried out at 37 °C for 1 h. The reaction was terminated by diluting in phosphate buffered saline and filtered through GF/C filters under vacuum. Bound radioactivity was determined by liquid scintillation spectrophotometry. Basal binding was assessed in the absence of agonist and non-specific binding was determined in the presence of 10  $\mu\text{M}$  GTP  $\gamma$  S. The percentage of [<sup>35</sup>S]GTP  $\gamma$  S bound was calculated as  $100 \times [\text{cpm}_{\text{sample}} - \text{cpm}_{\text{non-specific}}] / [\text{cpm}_{\text{basal}} - \text{cpm}_{\text{non-specific}}]$ .

### *Cyclic AMP assay and receptor desensitization*

Cells were challenged with N/OFQ or DPDPE in the presence of 1  $\mu\text{M}$  forskolin and 500  $\mu\text{M}$  1-methyl-3-isobutylxanthine (IBMX, Sigma) at 37 °C for 10 min. The reactions were terminated with 1 N perchloric acid and neutralized with 2N  $\text{K}_2\text{CO}_3$ . The cAMP level of each sample was determined using radioimmunoassay as described previously[17]. Data were averages from triplicate samples and presented as percentage of control (in the presence of forskolin alone). In the desensitization experiment, cells were prechallenged without or with 10  $\mu\text{M}$  of N/OFQ or 10  $\mu\text{M}$  [D-Pen<sup>2</sup>, D-Pen<sup>5</sup>]-enkephalin (DPDPE, Sigma) at 37 °C for 10 min. The cells were then washed twice with phosphate buffered saline and challenged again with 1  $\mu\text{M}$  of N/OFQ or 1  $\mu\text{M}$  DPDPE. The cellular cAMP levels were measured as above.

### *Measurement of extracellular metabolic rates*

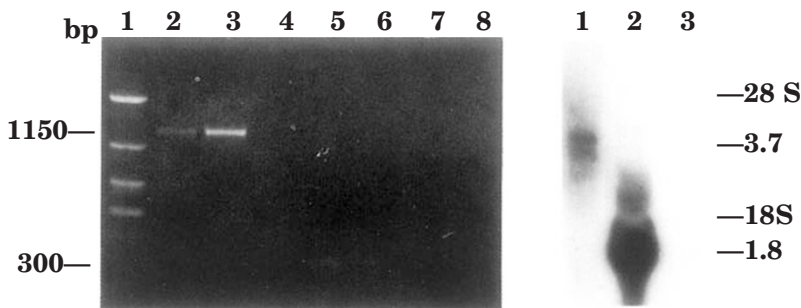
Extracellular metabolic rates were measured with the silicon-based light addressable potentiometer Cytosensor Microphysiometer (Molecular Devices Corp., CA, USA). CHO:hORL<sub>1</sub> cells was seeded at  $1 \times 10^5$  cells/capsule cups (Molecular Devices Corp.), and cultured overnight after the cells adhered to the capsule membrane. A spacer gasket and capsule insert (Molecular Devices Corp.) were placed into each capsule cup. The assembled capsule cups were loaded into Cytosensor Chambers. Low buffering and carbonate-free DMEM (Gibco-BRL) was used as medium in the Cytosensor, and the extracellular acidification rates were monitored, collecting a rate measurement every 90 sec. Cells were exposed to N/OFQ in running medium (0.1 ml/min) for 335 sec, and the effects on the extracellular acidification rate were measured by the instrument. The basal acidification rate was normalized to 100 %.

## RESULTS AND DISCUSSION

### *Endogenous expression of ORL<sub>1</sub> in human neuroblastoma cells*

In situ hybridization and immunohistochemistry studies revealed that ORL<sub>1</sub> and its endogenous ligand N/OFQ express widely over the brain[3, 4, 6, 9, 18, 19]. ORL<sub>1</sub> receptor transcripts are present in the CNS, including the hypothalamus, brainstem and spinal cord dorsal horn[1, 7, 8] as well as in a few peripheral organs such as intestine, vas deferens and spleen[6]. It suggests that ORL<sub>1</sub> plays a role in many central processes, including learning and memory. However, the endogenous expression of ORL<sub>1</sub> in any cell line has not been reported. Therefore, we examined several neuronal cell lines for the expression of ORL<sub>1</sub>. To detect ORL<sub>1</sub> receptor mRNA sensitively, we assessed the level of ORL<sub>1</sub> transcripts with RT-PCR. As shown in Fig 1A, with the primer sets of ORL<sub>1</sub>FBamH/ORL<sub>1</sub>REcoR and ORT5/ORT3, PCR products of expected sizes (1150 bp and 300 bp) were obtained, using cDNA reverse

transcripts from total RNA extracted from SH-SY5Y, or SK-N-SH cells. The PCR products were cloned, sequenced and confirmed as human ORL<sub>1</sub> cDNA. PCR reaction using cDNAs from CHO cells or genomic DNA from SK-N-SH cells as control template failed to produce any detectable PCR product. This result indicates the expression of ORL<sub>1</sub> in both human neuroblastoma cell lines (SH-SY5Y and SK-N-SH) tested. Furthermore, Northern analysis showed that a RNA species of 3.7 kb from SK-N-SH cells hybridized to the ORL<sub>1</sub> cDNA probe, confirming the expression of ORL<sub>1</sub> in SK-N-SH cells (Fig 1B, lane 1).



**Fig 1.** Expression of hORL<sub>1</sub> mRNA.

**A.** RT-PCR assay of total RNA isolated from human neuroblastoma cell lines. Lane 1: DNA markers, lane 2: SH-SY5Y, lane 3: SK-N-SH, lane 4: CHO, lane 5: SH-SY5Y, lane 6: SK-N-SH, lane 7: CHO, lane 8: genomic DNA of SK-N-SH. Lanes 2-4 were products from primer pair of ORL<sub>1</sub>FBamH / ORL<sub>1</sub>REcoR and lanes 5-7 of ORT5/ORT3. PCR products were 1150 and 300 bp respectively, as expected.

**B.** Northern analysis. Lane 1: SK-N-SH, lane 2: CHO:ORL<sub>1</sub>, lane 3: CHO. Each lane contains 20  $\mu$ g of total RNA.

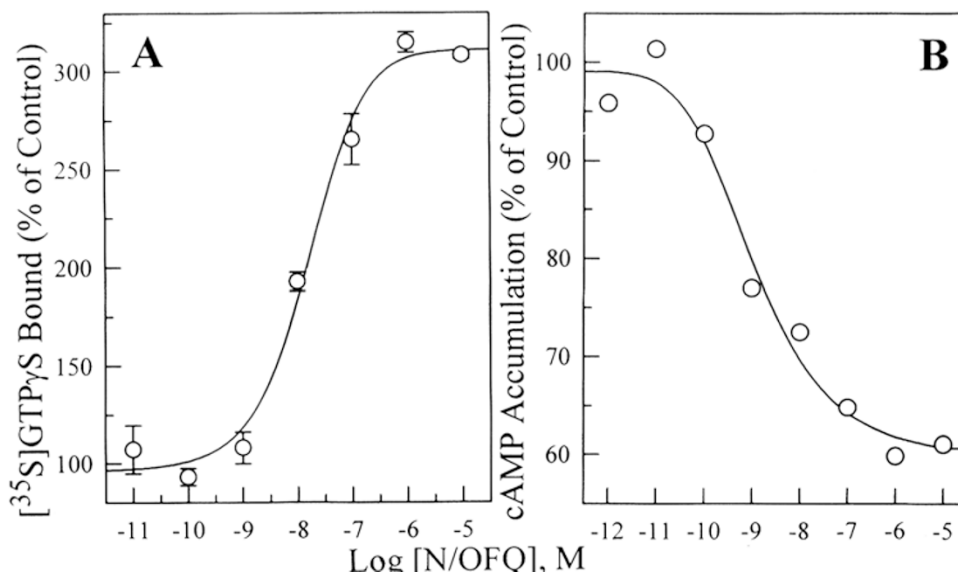
We demonstrated the presence of ORL<sub>1</sub> in SK-N-SH cells using radioligand saturation experiment. Our results showed that [<sup>3</sup>H]N/OFQ binds to the cell membranes from human neuroblastoma SK-N-SH cells specifically in a dose-dependent and saturable manner (data not shown). The above results clearly indicate that ORL<sub>1</sub> protein is expressed on the surface of neuroblastoma SK-N-SH cells. Our observation of the presence of ORL<sub>1</sub> in SH-SY5Y cells was independently confirmed by Houtani et al recently[20].

#### *ORL<sub>1</sub>-mediated signal transduction in SK-N-SH cells*

Activation of opioid receptors results in inhibition of adenylyl cyclase via activation of inhibitory G proteins. The ability for N/OFQ to attenuate cellular cAMP

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production has been demonstrated in CHO cells exogenously expressing ORL<sub>1</sub> [9, 10]. N/OFQ-stimulated activation of G proteins has also been shown in rat brain[11]. However, the inhibition of adenylyl cyclase activity mediated by ORL<sub>1</sub> has not been observed in neuronal cells and the signal transduction processes mediated by ORL<sub>1</sub> have not been systematically examined in a neuronal cellular environment. We have observed that SK-N-SH cells endogenously express ORL<sub>1</sub>. Therefore, we took on to determine the activation of the inhibitory G-protein stimulated by N/OFQ in SK-N-SH cells, using [<sup>35</sup>S]GTP  $\gamma$  S binding and cyclic AMP assays. Stimulation with N/OFQ caused significant increase of [<sup>35</sup>S]GTP  $\gamma$  S binding to the membranes from SK-N-SH cells. As shown in Fig 2A, N/OFQ stimulated [<sup>35</sup>S]GTP  $\gamma$  S binding was dose-dependent and saturable. The binding occurred at nanomolar concentration of the ORL<sub>1</sub> agonist and reached maximum around 10  $\mu$ M concentration with an EC<sub>50</sub> = 14  $\pm$  0.45 nM. Treatment of SK-N-SH cells with N/OFQ also resulted in



**Fig 2.** Activation of inhibitory G proteins by N/OFQ.

**A.** The dose-response curve of N/OFQ stimulated [<sup>35</sup>S]GTP  $\gamma$  S binding to membranes of SK-N-SH cells. Membranes were incubated with 8 nM [<sup>35</sup>S]GTP  $\gamma$  S, 40  $\mu$ M GDP, and with or without various concentrations of N/OFQ, and [<sup>35</sup>S]GTP  $\gamma$  S binding to membranes (EC<sub>50</sub> = 14  $\pm$  4.2 nM) was determined.

**B.** The dose-response curve for inhibition of forskolin-stimulated accumulation of cellular cAMP by N/OFQ. SK-N-SH cells were incubated with various concentrations of N/OFQ at 37  $^{\circ}$ C for 10 min, and the cAMP accumulation was determined. The EC<sub>50</sub> of N/OFQ inhibition was 0.80  $\pm$  0.45 nM. The figures are representatives of three independent experiments.

decrease of forskolin-stimulated accumulation of cAMP ( $EC_{50} = 0.80 \pm 0.45 \text{ nM}$ ), indicative of functionally negative coupling of ORL<sub>1</sub> to adenylyl cyclase in the neuronal cells (Fig 2B). The above data indicate that ORL<sub>1</sub> is functionally expressed in SK-N-SH cells.

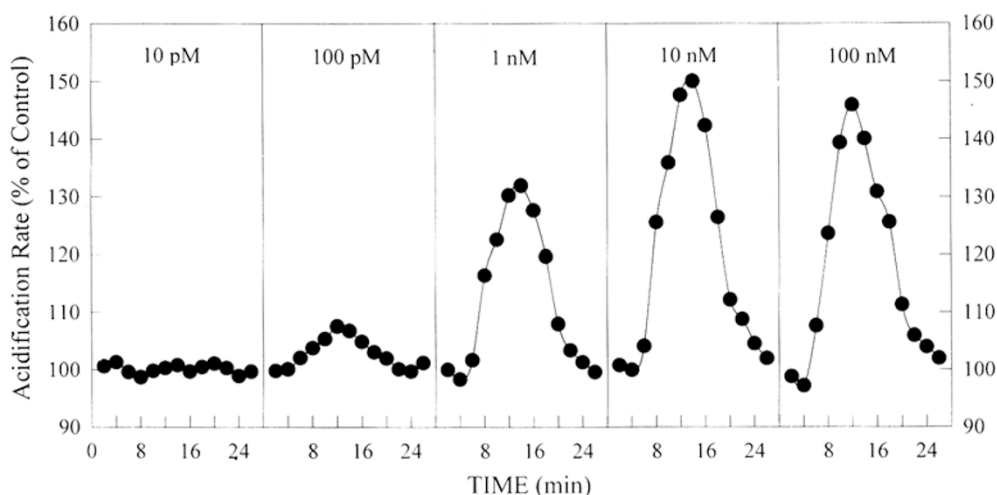
### *ORL<sub>1</sub>-mediated changes in the metabolic rates of CHO:hORL<sub>1</sub> cells*

In the above experiments, we have demonstrated, using human neuroblastoma cell line SK-N-SH, that endogenous ORL<sub>1</sub> mediates activation of G proteins and inactivation of adenylyl cyclase. Activation of ORL<sub>1</sub> receptor by N/OFQ was also accessed using microphysiometer.

Cytosensor microphysiometer measures the proton excretion of cells accurately in real time. Proton excretion, or extracellular acidification, depends largely on the energy consumption of a cell. When cell surface receptors are triggered by ligands, the cellular metabolism is activated by second messenger pathways resulting in increased energy metabolism and extracellular acidification which can be measured by microphysiometer.

To access cellular response to N/OFQ in real time scale by microphysiometry, a cell line stably expressed human ORL<sub>1</sub> was established. Human ORL<sub>1</sub> cDNA was cloned into expression vector pcDNA<sub>3</sub> and transfected into CHO cells. Transfected CHO cells stably expressing ORL<sub>1</sub> receptors were selected. A cell line CHO:hORL<sub>1</sub> with high expression of ORL<sub>1</sub> was selected and used in the following experiments. As detected in the Northern analysis, a RNA species at approximately 1.8 kb from the CHO:hORL<sub>1</sub> cell line strongly hybridized to the ORL<sub>1</sub> cDNA probe, indicating that the CHO:hORL<sub>1</sub> cell line expressed ORL<sub>1</sub> mRNA transcripts at high level. No hybridization signal was detected in untransfected CHO cells (Fig 1B).

Functional responses in CHO:hORL<sub>1</sub> cells was studied using a microphysiometer. Fig 3 shows the changes in extracellular acidification rates in CHO:hORL<sub>1</sub> cells upon application of various concentrations of ORL<sub>1</sub>-specific agonist N/OFQ. Infusion of N/OFQ into the chambers containing CHO:hORL<sub>1</sub> cells expressing N/OFQ receptors lead to a rapid increase in the extracellular acidification rate (Fig 3). The effect of N/OFQ on the extracellular acidification rate measured in the microphysiometer was found to be concentration dependent. The cellular response to N/OFQ occurred at 100 pmol/L concentration of N/OFQ and reached maximum at 10 nmol/L. The increase in metabolic rate stimulated by ORL<sub>1</sub> ligand peaked at approximately 150 % of the unstimulated rate. Pertussis toxin, a specific inhibitor of Gi/Go proteins, completely blocked the cellular response to N/OFQ (data not shown).



**Fig 3.** Changes in extracellular acidification rate (ECAR) in response to N/OFQ. Pump cycle used to perfuse the cells was 56 sec pump on/34 sec pump off, and the pump rate was set at 0.1 ml/min. The extracellular acidification rates were measured during the pump-off period. The rates were normalized to a 100 % ECAR prior to exposure to N/OFQ and plotted as a function of real time. Cells were exposed to various concentrations of N/OFQ as indicated for 335 sec.

In summary, we have demonstrated that ORL<sub>1</sub> is functionally expressed in human neuroblastoma SK-N-SH cells. RT-PCR and Northern analysis indicate the presence of ORL<sub>1</sub> transcripts in SK-N-SH and SH-SY5Y cells. [<sup>3</sup>H]N/OFQ bound to the membranes of SK-N-SH cells specifically. Binding of the receptor with its specific agonist N/OFQ activated inhibitory G-protein as indicated by stimulation of [<sup>35</sup>S]GTP  $\gamma$  S binding and inhibition of forskolin-stimulated accumulation of cAMP. Treatment of the cells with N/OFQ, the specific ligand of ORL<sub>1</sub>, resulted in an increase in the extracellular acidification rate in a concentration-dependent manner.

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## REFERENCES

- [1] Reisine T. Opiate receptors. *Neuropharmacology* 1995; **34**:463-72.
- [2] Mollereau C, Parmentier M, Mailleux Pet al. ORL<sub>1</sub>, a novel member of the opioid receptor family Cloning, functional expression and localization. *FFBS Letters* 1994; **341**:33-8.
- [3] Fukuda K, Kato S, Mori K, et al. cDNA cloning and regional distribution of a novel member of the opioid receptor family. *FEBS Letters* 1994; **343**:42-6.
- [4] Chen Y, Fan Y, Liu J, et al. Molecular cloning, tissue distribution and chromosomal localization of a novel member of the opioid receptor gene family. *FEBS Letters* 1994; **347**:279-83.
- [5] Bunzow JR, Saez C, Mortrud M, et al. Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a  $\mu$ ,  $\delta$  or  $\kappa$  opioid receptor type. *FEBS Letters* 1994; **347**:284-8.
- [6] Wang JB, Johnson PS, Imai Y. DNA cloning of an orphan opiate receptor gene family member and its splice variant. *FEBS Letters* 1994; **348**:75-9.
- [7] Wick M J, Minnerah SR, Lin X, Elde R, Law PY, Loh HH. Isolation of a novel cDNA encoding a putative memberane receptor with high homology to the cloned  $\mu$ ,  $\delta$  or  $\kappa$  opioid receptors. *Mol Brain Res* 1994; **27**:37-44.
- [8] Lachowicz JE, Shen Y, Monsma F J, Jr, Sibley DR. Molecular cloning of a novel G protein-coupled receptor related to the opioid receptor family. *J Neurochem* 1995; **64**:34-40.
- [9] Meunier J-C, Mollereau C, Toll L. Isolation and structure of the endogenous agonist of opioid receptor-like ORL<sub>1</sub> receptor. *Nature* 1995; **377**:532-5.
- [10] Reinscheid R., Nothacker H.-P, Bourson A, et al. Orphanin FQ: a neuropeptide that activates an opioid-like G protein-coupled receptor. *Science* 1995; **270**:792-4.
- [11] Sim L J, Xiao R, Childers SR. Indentification of opioid receptor-like (ORL<sub>1</sub>) peptide-situlated [<sup>35</sup>S] GTP gamma S binding in rat brain. *Neuro Report* 1996; **7**:729-33.
- [12] Vaughan CW, Christie MJ. Increase by the ORL<sub>1</sub> receptor (opioid receptor-like<sub>1</sub>) ligand, nociceptin, of inwardly rectifying K conductance in dorsal raphe nucleus neurones. *Brit J Pharmacol* 1996; **117**:1609-11.
- [13] Hochhaus G, Yu VC, Sadee W, Delta opioid receptors in human neuroblastoma cell lines. *Brain Res* 1986; **382**:327-31.
- [14] Church, Gilbert, Genomic sequencing. *PNAS* 1984; **81**:1991-5.
- [15] Velu T J, Beguinot L, Vas WC, Zhang K, Pastan I, Lowy DR. Retroviruses expressing different levels of the normal epidermal growth factor receptor: biological properties and new bioassay. *J Cell-Biochem* 1989; **39**:153-66.
- [16] Pei G, Kieffer BL, Lefkowitz RJ, Neil JF. Agonist-dependent phosphorylation of the mouse  $\delta$ -opioid receptor: involvement of G protein-coupled receptor kinasas but not protein kinase C. *Mol Pharmacol* 1995; **48**:173-7.
- [17] Cai YC, Zhang Y, Wu Y, Pei G.  $\delta$  opioid receptor in neuronal cell undergoes acute and homologous desensitization. *Biochem Biophys Res Comm* 1996; **219**:342-7.
- [18] Anton B, Fein J, To T, Li X, Silberstein L, Evens C. Immunohistochemical localization of ORL<sub>1</sub> in the central nervous system of the rat. *J Comp Neurol* 1996; **368**:229-51.
- [19] Houtani T, Nishi M, Takeshima H, Nukada T, Sugimoto T. Structure and regional distribution of nociceptin/orphanin FQ precursor. *Biochem Biophys Res Comm* 1996; **219**:714-9.
- [20] Connor M, Yeo A, Henderson G. The effect of nociceptin on Ca<sup>2+</sup> channel current and intracellular Ca<sup>2+</sup> in the SH-SY5Y human neuroblastoma cell line. *Br J Pharmacol* 1996; **118**:205-7.

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