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Functional expression of opioid receptor-like receptor and its endogenous specific agonist nociceptin/orphanin FQ during mouse embryogenesis

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ABSTRACT

Expression of opioid receptor-like receptor (ORL_1) and its endogenous peptide agonist nociceptin/orphanin FQ (N/OFQ) during mouse embryogenesis have been investigated. Transcripts of ORL_1 and N/OFQ were detected by RT-PCR in mouse brain of day 8 embryo (E8) and the expression continued afterwards. Northern blot analysis revealed abundant expression of ORL_1 at postnatal day 1 (P1) and N/OFQ at E17 and P1 in the brain but none was detected in other embryonic tissues. The presence of functional ORL_1 in mouse embryonic brain was also confirmed by specific binding of [^{3}H] N/OFQ (kd=1.3 ± 0.5 n*M* and Bmax = 72 ± 9 *fmol*/mg protein) as well as by N/OFQ-stimulated G protein activation.

Key words: *Opioid receptor-like receptor (ORL_1), nociceptin/orphanin FQ (N/OFQ), mouse embryogenesis, functional expression.*

INTRODUCTION

Opioid receptors belong to the G-protein-coupled receptor family that is characterized by the seven transmembrane spanning domains in structure. Three subtypes of the opioid receptors (μ, δ, and κ) have been cloned and characterized through their

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distinct affinities for different opioid ligands. These opioid receptors are all coupled to the inhibitory G protein (Gi) and negatively regulate adenylate cyclase[1]. Opioid receptor-like receptor (ORL_1), a new member of this family of G-protein coupled receptors, has been cloned from brain recently, which shares high homology in sequence with other opioid receptors[2-8]. ORL_1 binds to previously identified opioid peptides with poor affinity, and its endogenous specific agonist nociceptin/orphanin FQ (N/OFQ) has just been identified[9, 10]. Data from our and other laboratories have demonstrated that in neuronal cells, N/OFQ inhibits forskolin-stimulated cAMP accumulation, stimulates activation of pertussis toxin-sensitive G proteins[11-13], and increases inwardly rectifying K^+ conductance[14]. Behavioral studies demonstrate that N/OFQ, unlike other opioids, produces hyperalgesic effect[10] and even functionally antagonizes the antinociceptive effect of other opioids[15, 16]. It was lately found that ORL_1-knock-out mice display hearing impairment[17]. However, information on the expression of ORL_1 and N/OFQ during mouse embryonic development is lacking. In the present study, we have demonstrated that both ORL_1 and N/OFQ express in mouse embryonic brain but not in other tissues and that N/OFQ stimulation leads to activation of inhibitory G proteins.

MATERIALS AND METHODS

Animals

Pregnant female Balb/c mice on embryonic days 8, 10, 14, 17, 19 (E8, E10, E14, E17, E19), postnatal day 1 (P1) mice and adult mice were obtained from the Animal House of Shanghai Institute of Cell Biology. All animals were sacrificed with the use of cervical dislocation following the Guideline for the Care and Use of Animals approved by the Institute.

Isolation of total RNA

50-100 mg of tissue was homogenized in 1 ml of TRIzol Reagent (Gibco-BRL) using a glass homogenizer. Total RNA was extracted according to the manufacturer-provided protocols and dissolved in diethypyrocarbonate (DEPC) treated water. The amount of total RNA was determined by absorbence at 260 nm.

Reverse transcription polymerase chain reaction(RT-PCR)

First-strand cDNA was made from total RNA by using Superscript preamplifiaction system (Gibco-BRL) and following the procedures suggested by the manufacturer. 2 μg of total RNA and 0.5 μg of oligo $(dT)_{12\text{-}18}$ was heated to 70 ℃ for 10 min in 11 μl of DEPC-treated water. 4 μl of 5 $\times$ synthesis buffer (250 m*M* Tris-HCl, pH 8.3, 375 m*M* KCl, 15 m*M* $MgCl_2$), 1 ml of 10 m*M* dNTP mix, 2 μl of 0.1 *M* DTT, 1 μl of RNasin (20U/μl) and 1 μl of reverse transcriptase (SuperScript II RT; 200U/μl) were added to the sample, and then incubated the samples at 42 ℃ for 1 h. The reaction was terminated by incubating the mixture at 70 ℃ for 10 min[18].

PCR primers used were ORT5 (GGGATCTCCACCAGGCACTCGATC) and ORL_1REcoR (CAGGAATTCCATGGGCAGGTCCACGCCTAGTC) for ORL_1; OFQ5 (ACTGCCTCACCTGCCAGG) and OFQ3 (GGCTCCTTCTGGCTACAC) for N/OFQ. ORT5/ORL_1 REcoR primers correspond to regions from bases 497 to 515 and 1293 to 1310 on human ORL_1 cDNA[2] respectively and are conserved among rat, mouse and human. OFQ5/OFQ3 primers were correspond to regions from bases 31 to 47 and 502 to 518 on rat N/OFQ precursor cDNA[9] respectively. The PCR reactions (50 μl) contained 5 μl of first-strand cDNA, 2 units of Taq polymerase (Promega). The condi-

tions used were: 94 ℃ for 1 min; 60 ℃ for 1 min; 72 ℃ for 1.5 min. This cycle was repeated 30 times. The PCR products (～ 800 bp and 480 bp respectively) were analyzed on a 1% agarose gel.

Northern blot analysis

Total RNA of each sample was electrophoresed on a 1% agarose-glyoxal gel (30 μg/lane except lane 1 was 10 μg), transferred onto a nylon membrane (Amersham) with 20 × SSC (1 × 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).

The 1150 bp restriction fragment containing entire coding region of ORL_1 was released from plasmid $pcDNA_3$:$hORL_1$[18], purified using QIAEX Gel Extraction Kit (QIAEX), and used as ORL_1 probe. N/OFQ probe was the 480 bp PCR product of OFQ5/OFQ3 purified using QIAEX Gel Extraction Kit. ^{32}P-labeled random-primed probes were made to a specific activity of 5×10^8 dpm/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 % Sodium Dodecyl Sulfate (SDS), 1 m*M* EDTA at 65 ℃ for 4 h, and hybridized to ^{32}P-labeled probe at 65 ℃ for 20 h[19]. The membrane was washed twice in 2 × SSC/0.1% SDS at room temperature for 15 min each, and then twice in 0.5 × SSC/0.1% SDS at 55 ℃ for 15 min each. The membrane was exposed to X-ray film (Kodak) for 5-7 days.

N/OFQ binding assay

Mouse embryonic brain membranes were prepared by homogenizing the embryonic brain in 1 ml of lysis buffer containing 5 m*M* Tris-HCl (pH 7.5), 5 m*M* EDTA, and 5 mM EGTA and the membrane lysate was centrifuged at 3000 × g at 4 ℃ for 15 min. Then the supernatant was centrifuged at 30,000 × g at 4 ℃ for 30 min. Pellets were solubilized in buffer containing 50 m*M* Tris-HCl (pH 7.5), 5 m*M* EDTA, and 5 m*M* EGTA. The binding assay was carried out basically as described[11]. Aliquot with different concentrations (2.0 ～ 11.0 n*M*) of [^{3}H] N/OFQ (39 Ci/mmol, Phoenix Pharmaceuticals, Inc, CA) in a total volume of 0.2 ml were incubated at 30 ℃ for 60 min. The reaction was terminated by diluting with cold phosphate-buffered saline and filtered through glass fiber filters under vacuum. Bound radioactivity was measured by liquid scintillation counter (Beckmann Instruments, Torrance, CA). Nonspecific binding was measured in the presence of 100 mM etorphine (Sigma). Equilibrium binding data were analyzed using the curve- fitting program LIGAND. Protein content of each sample was determined as described above.

[^{35}S]GTPS binding assay

The assay was carried out as described[11, 20]. Embryonic brain membranes were prepared as above. The membrane (containing 12 μg protein) in 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 m*M* DTT, 100 m*M* NaCl, 40 μM GDP, and 0.5 nM [^{35}S]GTP γ S (1200 Ci/mmol, DuPont-New England Nuclear) in the presence or absence of N/OFQ, [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (DAGO) (Sigma), or UK14304 (Sigma) in a total volume of 100 μl were incubated at 30 ℃ for 60 min. The reaction was terminated by adding 4 ml phosphate-buffered saline and then immediately filtered through GF/C filters under vacuum. The filters were washed and counted by liquid scintillation counter. Data were means of duplicate samples. Basal binding was determined in the absence of agonist and non-specific binding was obtained in the presence of 10 mM GTP γ S. The percentage of stimulated [^{35}S]GTP γ S was calculated as $100 \times (cpm_{sample} - cpm_{nonspecific}) / (cpm_{basal} - cpm_{non\text{-}specific})$.

RESULTS AND DISCUSSION

Expression of ORL_1 and N/OFQ during development

In situ hybridization and immunohistochemistry studies revealed that ORL_1 and

its endogenous ligand N/OFQ express widely over the brain[3, 4, 6, 9, 21, 22]. ORL_1 transcripts are present in the CNS, including the hypothalamus, brainstem and spinal cord dorsal horn[1, 7, 8]. ORL_1 also expresses in a few peripheral organs such as intestine, vas deferens and spleen[6]. It suggests that ORL_1 plays a role in many central processes, including learning and memory. Transcripts of N/OFQ precursor protein was detected during mouse embryogenesis[23]. However, the expression of ORL_1 in embryogenesis has not been reported. Therefore, we examined the expression of ORL_1 and N/OFQ in mouse brains on various embryonic days (E8, E10, E14, E17, E19) and postnatal day 1 (P1) as well as in adult mouse brain. To detect ORL_1 receptor mRNA sensitively, we measured the levels of ORL_1 and N/OFQ transcripts with RT-PCR and Northern blotting. As shown in Fig 1A and B, with the primer sets of ORT5/ORL_1REcoR and OFQ5/OFQ3, PCR products

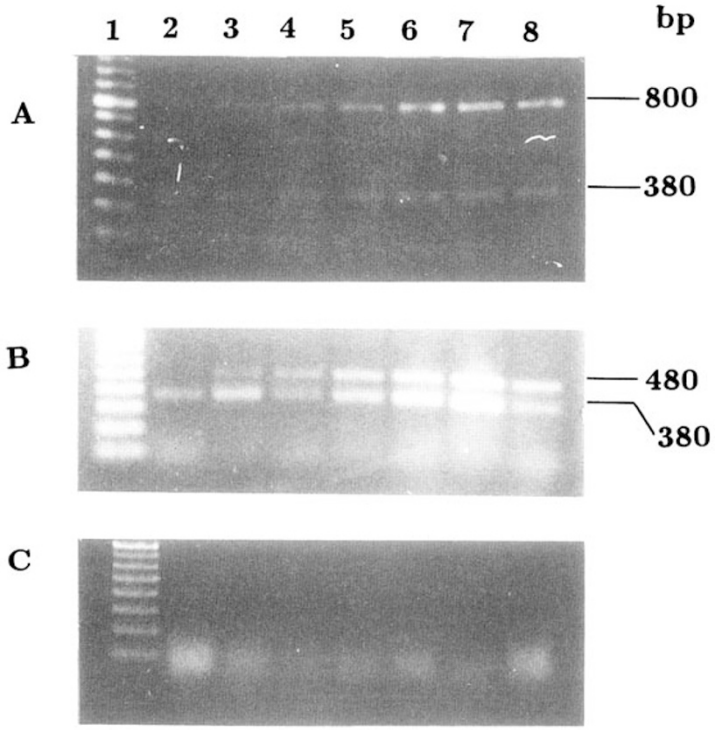

Fig 1. Embryonic expression of ORL_1 and N/OFQ mRNA in mouse brain detected by RT-PCR. The expression was assayed by RT-PCR for ORL_1 using primer pair of ORL5/ORL_1REcoR (A) and for N/OFQ using primer pair of OFQ5/OFQ3 (B). As control, the total RNA was used as the PCR template without RT reaction, using primer pair of ORL5/ORL_1REcoR and OFQ5/OFQ3 (C). Internal controls of actin was included in each PCR reaction and the actin products were shown as a band of 380 bp. Lane 1: DNA markers, lane 2:E8 (0.3; 0), lane 3: E10 (1.0; 0.1), lane 4:E14 (2.7; 0.9), lane 5:E17 (5.1; 0.95), lane 6:E19 (9.8; 0.90), lane 7: P1 (9.9; 0.92), lane 8: adult (9.5; 1.1). In each lane, the amount of PCR product determined by densitometer relative to internal control β-actin was indicated in parentheses (A; B).

of expected sizes (800 bp and 480 bp) were detected in brain samples from E10 to adult mice (Lanes 3-8). PCR reactions using embryonic brain RNA (without reverse transcription Fig 1C) and the cDNA form other embryonic tissues (kidney, liver, heart) as template failed to produce any detectable PCR product (data not shown). β-actin was used as a positive internal control in PCR ($\sim$ 380bp). The quantitative densitometry analysis show from mouse E10 to E19, the amounts of RT-PCR products (relative to the product of β-actin used as an internal control) of ORL_1 and OFQ increased and they maintained the similar levels since E19. The above results indicate that the transcripts for ORL_1 and N/OFQ are present in embryonic, postnatal and adult mice brains.

In Northern analysis, ORL_1 and N/OFQ transcripts were detected only in embryonic and postnatal mice brains, not in other embryonic and postnatal tissues. For ORL_1, P1 brain contains a RNA species of 3.7 kb, hybridizable to the ORL_1 cDNA probe (Fig 2A, lane 3), but ORL_1 transcripts were not detectable in E17 under the same conditions (Fig 2A, lane 2). For N/OFQ, a RNA species of 1.3 kb were detected (Fig 2B, lane 2, 3) in both E17 and P1 brain.

The results of RT-PCR and Northern analysis indicate that the transcripts of ORL_1 and N/OFQ are present on and after E10. Nothacker et al reported that the OFQ precursor mRNA was detected in fetal human brain and kidney but not in adult human kidney by Northern analysis[24]. In present study, the expression of OFQ did not been detected in mouse E17 and P1 kidney. This could be a result of the samples used in different species and/or different embryonic stages. In μ receptor-deficient mice, some unexpected changes in sexual function in male homozygotes were observed, such as reduced mating activity, a decrease in sperm count and motility, and litter size[25]. These data suggest that opioid receptor may play an important role in development. Other reports confirmed that N/OFQ mRNA was detected in mouse brains of embryonic day 14, and larger amounts were detected on postnatal day 1. Then the levels of mRNA decreased gradually as mouse grew[23].

Membrane fractions were prepared from E17 brain, and the presence of ORL_1 receptors was examined using [^{3}H] N/OFQ, a specific agonist of ORL_1. As shown in Fig 3A, binding of N/OFQ to embryonic brain membranes was specific and saturable. Analysis of saturation binding data indicates a dissociation constant kd of 1.3 $\pm$ 0.5 n*M* and B_{max} of 72 $\pm$ 9 fmol/mg protein, comparable to the value obtained in rat brain homogenates[26] and in neuroblastoma cells[11, 13]. These data are consistent with our RT-PCR and Northern results and confirm that the gene product of ORL_1 is expressed on cell membranes of embryonic brain.

ORL_1-mediated G protein activation

[^{35}S]GTP-γS binding is an effective method to probe gonist-dependent activation of G proteins. Using this method, the activation of pertussis toxin (PTX)-sensitive G proteins after agonist occupation of opioid[27], muscarinic[28, 29], α2-adrenergic

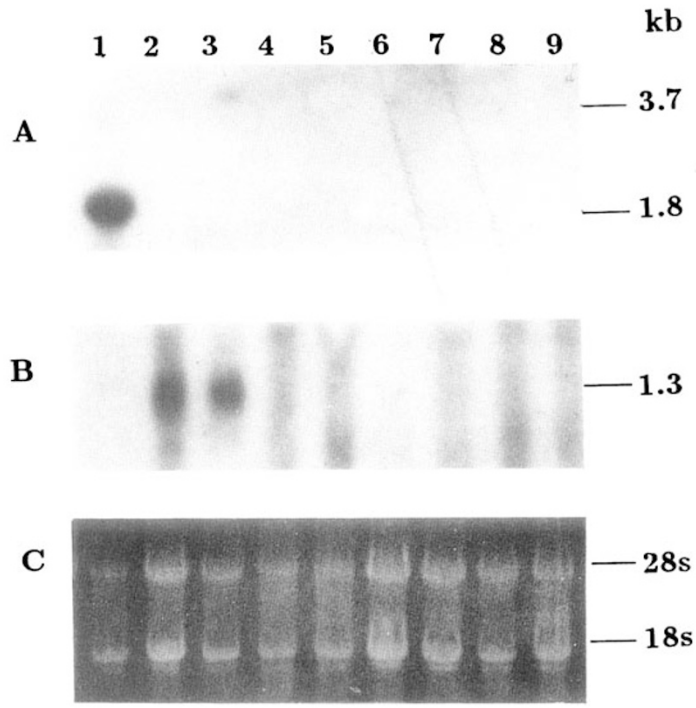

Fig 2. Northern analysis of ORL_1 (A) and N/OFQ (B) mRNA transcripts. Lane 1: CHO-ORL_1 cells; lanes 2 and 3, brain; lanes 4 and 5, kidney; lanes 6 and 7, liver; lanes 8 and 9, heart. Lanes 2, 4, 6, 8: E17; lanes 3, 5, 7, 9: P1. (C) Ethidium bromide staining of the gel before Northern blotting.

plasma membrane-bound receptors have been determined[13]. We have observed that ORL_1 receptor was expressed on mouse embryonic brain membranes. Therefore, we took on to determine the activation of the inhibitory G-protein mediated by ORL_1 in embryonic brain using [^{35}S]GTP γ S binding. As shown in Fig 3B, activation of μ opioid receptor by DAGO and α 2-adrenergic by UK14304 increased [^{35}S]GTP γ S binding 10 - 30 %, however, in strong contrast, stimulation of 10 m*M* N/OFQ caused an 100 % increase in [^{35}S]GTP γ S binding to the membranes from embryonic brain. These data confirm the expression of ORL_1 in embryonic brain and indicate that ORL_1 is functionally coupled to G protein. The results also suggest that ORL_1 may be present in embryonic brain more abundant than μ opioid receptors, or alternatively, it may couple to G protein more efficiently than μ opioid receptor in fetal brain. Experimental data have shown that ORL_1 and μ opioid receptor mediate opposite effects in pain modulation[9, 10]. Changes in sexual functions such

as reduced mating activity, decreases in sperm count and motility, and litter size have been observed in male homozygotes of μ receptor-knock-out mice[25]. These results suggest that opioid receptor may play a role in development. The differential activities in G protein activation mediated by these two important receptors may therefore have developmental significance.

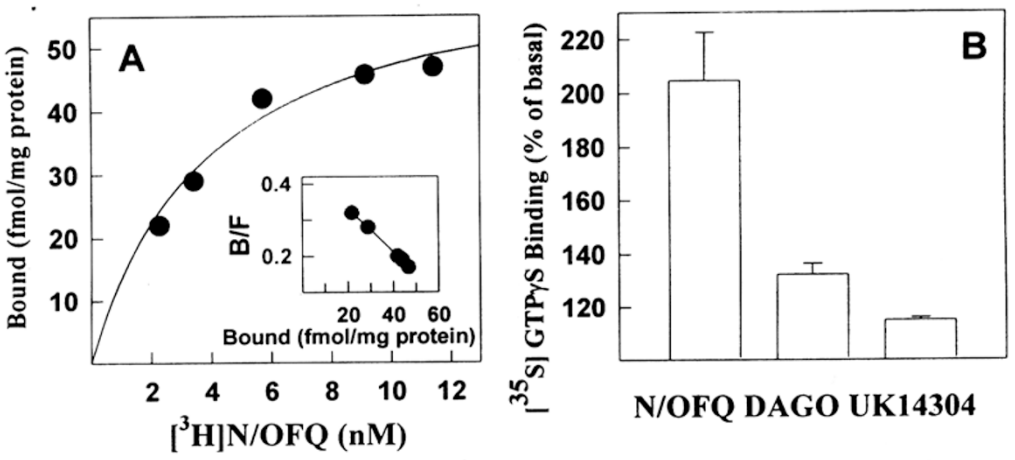

Fig 3. Functional expression of ORL_1 in mouse embryonic brain. (A) Saturation binding of [^{3}H]-labeled N/OFQ to ORL_1 receptor. The membrane preparations were incubated with various concentration of [^{3}H] N/OFQ and the [^{3}H]N/OFQ bound was determined. (B) [^{35}S]GTP ɣ S binding assay: Assays were performed in the presence of 10 mM N/OFQ, 10 μM DAGO, or 10 μM UK14304 respectively, at 30 ℃ for 60 min. Data were means ± standard error of two independent experiments performed in duplicate.

In summary, we have demonstrated that ORL_1 and N/OFQ is functionally expressed in mouse embryonic brain. RT-RCR and Northern analysis indicate the presence of ORL_1 and N/OFQ transcripts in mouse embryonic brain. [^{3}H]N/OFQ bound to the embryonic brain membranes specifically. Binding of the receptor with its specific agonist N/OFQ activated inhibitory G protein as indicated by stimulation of [^{35}S]GTP ɣ S binding.

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