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# Ror1-Ror2 COMPLEXES MODULATE SYNAPSE FORMATION IN HIPPOCAMPAL NEURONS

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**Abstract—Ror1 and Ror2, a small family of tyrosine kinase receptors, have been implicated in multiple aspects of brain development in *C. elegans* and *X. laevis*. More recently, we have shown that these receptors modulate the rate of neurite elongation in cultured rat hippocampal neurons. However, no information is available regarding a potential role of these receptors in other developmental milestones in mammalian central neurons. Neither is the identity known of the Ror ligand(s) and/or the signal transduction pathway(s) in which they participate. Here we report that the down regulation of either Ror1 or Ror2 led to a significant decrease in synapse formation in cultured hippocampal neurons. Simultaneous targeting of Ror proteins, however, did not result in an additive phenotype. Our results also indicated that Ror1 and Ror2 physically interact in the mouse brain, suggesting that they might function as heterodimers in central neurons. In addition, these Ror complexes interacted with Wnt-5a mediating its effects on synaptogenesis. Together, these data suggest that Ror proteins play a key role in Wnt-5a-activated signaling pathways leading to synapse formation in the mammalian CNS.** 

**Key words: Ror1, Ror2, synaptogenesis, siRNAs, antisense oligonucleotides.**

Synapse assembly requires the coordinated activity of multiple extracellular cues and intracellular response machineries linked by cell surface receptors. In the peripheral nervous system, many of the molecular players that regulate synaptogenesis are well characterized (Sanes and Lichtman, 2001). At the level of the muscle cell membrane, environmental signals such as agrin and neuregulin I are transduced by the muscle-specific tyrosine kinase receptor (MuSK) and erbB, respectively, to elicit the formation of neuromuscular junctions (Gautam et al., 1996; Glass et al., 1996; Buonanno and Fischbach, 2001). On the other hand, the molecular mechanisms underlying synapse formation among central neurons are poorly understood. Although recent evidence implicated other members of the superfamily of receptor tyrosine kinases (RTKs) in synapse formation in the CNS, a detailed picture of the mosaic of receptors that are involved in the formation of central synapses is far from complete (Garner et al., 2002; Huang and Reichardt, 2003; Salinas, 2003; Klein, 2004; Umemori et al., 2004).

A recently discovered family of RTKs, the Ror family, has been implicated in brain development based on data obtained in *C. elegans* and *X. laevis* (Forrester et al., 1999, 2004; Koga et al., 1999; Hikasa et al., 2002). These receptors have also been detected in the mammalian CNS. Analysis of their pattern of expression showed that Ror1 and Ror2 levels increased as central neurons developed either *in situ* or in culture (Oishi et al., 1999; McKay et al., 2001; Paganoni and Ferreira, 2003). This pattern of expression suggested a role for Ror proteins in neurite elongation and synapse formation, two developmental processes essential for the formation of a mature neuronal network. Furthermore, localization studies indicated that Ror1 and Ror2 were highly enriched in dendrites of cultured central neurons (McKay et al., 2001; Paganoni and Ferreira, 2003). This subcellular localization positions them ideally to transduce signals necessary for postsynaptic differentiation leading to synapse formation in the CNS. However, no direct evidence is available regarding the role of Ror1 and Ror2 in the formation of synapses and/or the identity of their ligand(s) in mammalian central neurons.

In this study, we suppressed Ror1 and Ror2 expression by means of RNA interference (RNAi) and antisense oligonucleotides in cultured hippocampal neurons. Our results showed that the down regulation of Ror1 and/or Ror2 led to the formation of fewer synaptic contacts as compared to Ror-expressing controls. Furthermore, we provided evidence suggesting that Ror1 and Ror2 might function as heterodimers in the Wnt-5a signaling pathway in the mammalian brain.

## EXPERIMENTAL PROCEDURES

### Preparation of hippocampal cultures

Neuronal cultures were prepared from the hippocampi of embryonic day 16 (E16) mouse embryos as previously described (Goslin and Banker, 1991). In brief, embryos were removed and their hippocampi dissected and freed of meninges. The cells were dissociated by trypsinization (0.25% for 15 min at 37 °C) followed by trituration with a fire-polished Pasteur pipette and plated onto poly-L-lysine-coated coverslips or 60 mm tissue culture dishes in minimum essential medium (MEM) with 10% horse serum (Invitrogen, Carlsbad, CA, USA). Coverslips were then transferred to dishes containing an astroglial monolayer and maintained in MEM containing N2 supplements (Bottenstein and Sato, 1979) plus ovalbumin (0.1%) and sodium pyruvate (0.1 mM). For biochemical experiments, the medium was replaced with glia-conditioned MEM containing N2 supplements (Bottenstein and Sato, 1979) plus ovalbumin (0.1%) and sodium pyruvate (0.1 mM). The Northwestern University Animal Care and Use Committee approved this

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*Abbreviations:* E16, embryonic day 16; HEK293, Human embryonic kidney; MEM, minimum essential medium; MUT, mutated; PBS, phosphate buffer saline; RTKs, receptor tyrosine kinases; SCR, scrambled; siRNAs, small interfering RNAs.

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experimental protocol in accordance with USPHS regulations and applicable federal and local laws. Neuronal cultures were prepared following established protocols to minimize the number of animals used and their suffering.

### Small interfering RNA preparation and transfection

Small interfering RNAs (siRNAs) corresponding to Ror1 and Ror2 were designed as described elsewhere (Paganoni and Ferreira, 2005). The following target sequences were used: Ror1 5′-AATCTCCTTCCGGGCAACCAA-3′ and Ror2 5′-AAGATTCGGAGGCAATCGACA-3′, corresponding to nucleotides 312–332 and 107–127 of the Ror1 or Ror2 mouse cDNAs, respectively (Oishi et al., 1999). As controls, scrambled siRNAs ("siRNA SCR") and siRNAs carrying a two-base pair change ("siRNA MUT") were used (Ror1-SCR: 5′-AACCTGCGACCTAGCTCCTAA-3′; Ror2-SCR: 5′-AATGTCACAGATAAGCGAGCG-3′; Ror1-MUT: 5′-AATCTCCTTCCGAACAACCAA-3′; Ror2-MUT: 5′-AAGATTCGGAAACAATCGACA-3′).

Transfections were carried out in 60 mm tissue culture dishes 4 days after plating using the TransMessenger transfection reagent (Qiagen, Germantown, MD, USA) as previously described (Paganoni and Ferreira, 2005). Briefly, transfection complexes were prepared by combining siRNA duplexes, Enhancer R and Transmessenger reagent in buffer EC-R (Qiagen). Complexes were then diluted in 2 ml of warm MEM containing N2 supplements (final siRNA concentration: 100 nM). Cultures were incubated in this medium for 3 h and then returned to the medium in which they were growing before transfection. Analysis was performed 72 h later.

### Antisense treatments

Oligonucleotides were designed to target the 5′ end of the mouse Ror1 and Ror2 cDNAs and were synthesized by Biosource International (Camarillo, CA, USA) as previously described (Paganoni and Ferreira, 2005). The following 18-mer antisense oligonucleotides (AS) were used in this study: for Ror1 5′-CTGTTTCTTGGGCATCAG-3′ and for Ror2 5′-CACAGAGGCACACGGCTC-3′, corresponding to nucleotides+80+97 and +24+41 of the Ror1 and Ror2 mouse cDNAs, respectively (Oishi et al., 1999). Controls were treated with sense (S) oligonucleotides (5′-CTGATGCCCAAGAAACAG-3′ for Ror1 and 5′-GAGCCGTGTGCCTCTGTG-3′ for Ror2). All oligonucleotides contained phosphorothioate groups at the last three residues in the 3′ terminal region (Ferreira, 1999).

Sense and antisense oligonucleotides were added directly to the media of hippocampal cultures from day 4 to day 7 after plating every 12 hours at a 50 $\mu$M (first treatment) or 25 $\mu$M (following treatments) final concentrations. Cells were harvested for analysis 7 days after plating.

### Plasmid transfections

Human embryonic kidney (HEK293) cells were maintained in MEM plus 10% horse serum (Invitrogen) and grown at 37 °C in 5% $CO_2$ and 90% humidity. Cultures were fed twice a week and passaged when 80% confluent. Cells were renewed every 10 passages.

Expression plasmids encoding mouse Ror1 or Ror2 (pcDNA3-mRor1-Flag, pcDNA3-mRor2-Flag and pcDNA3-mRor2-HA, a generous gift from Dr. Minami, Kobe University, Japan) and expression plasmids encoding HA-tagged mouse Wnt-5a (Upstate, Lake Placid, NY, USA) were transfected into HEK293 cells using the Nucleofector™ apparatus (Amaxa, Gaithersburg, MD, USA). For each reaction, one million HEK cells were resuspended in 100 $\mu$l Nucleofector solution containing 10 $\mu$g DNA and electroporated using program Q-01. Cells were plated in MEM plus 10% horse serum and processed 72 h after plating.

Transfection of HA-tagged Wnt-5a into astrocytes was performed using the Nucleofector™ apparatus (Amaxa) as previously described (Paganoni and Ferreira, 2005). Briefly, confluent astrocyte cultures were trypsinized (0.25% for 15 min at 37 °C), pelleted down (10 min at 1000 rpm) and resuspended in Amaxa glia nucleofector solution, transferred to an electroporation cuvette and "nucleofected" according to the manufacturer's protocol (program T-20). For each reaction, four million astrocytes and 3 $\mu$g of cDNA were used. Astrocytes were then plated at a density of 40,000 cells/cm$^2$ and allowed to grow for 48 h. Transfected and untransfected astrocyte monolayers were then used to set up co-cultures with hippocampal neurons as described above. For some experiments, we collected the media in which untransfected and Wnt-5a-transfected astrocytes had been maintained for 7 days. The media were filtered to remove cell debris before being added directly to untreated hippocampal neurons and to neurons in which Ror1 and/or Ror2 expression has been suppressed using specific antisense oligonucleotides as described above.

### Immunocytochemistry

Hippocampal neurons and HEK cells were fixed for 20 min with 4% paraformaldehyde in phosphate buffer saline (PBS) containing 0.12 M sucrose. They were then permeabilized in 0.3% Triton X-100 in PBS for 5 min and rinsed twice in PBS. The coverslips were preincubated in 10% bovine serum albumin (BSA) in PBS for 1 h at room temperature and exposed to the primary antibodies (diluted in 1% BSA in PBS) overnight at 4 °C. Finally, the cultures were rinsed in PBS and incubated with secondary antibodies for 1 h at 37 °C. The following primary antibodies were used: anti-$\alpha$-tubulin (clone DM1A, 1:200, Sigma, St Louis, MO, USA), anti-Ror1 and anti-Ror2 (1:20, Paganoni and Ferreira, 2003), monoclonal anti-synaptophysin (clone SY38, 1:50, Millipore Bioscience Research Reagents, Belmont, MA, USA), polyclonal anti-synaptophysin (SYP (H-93), 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-synapsin I (1:100, Zymed, San Francisco, CA, USA), anti-microtubule associated Protein 2 (MAP 2) (1:100, clone AP-14; Caceres et al., 1984), anti-peptide DYKDDDK (Flag) (1:1000, Millipore Bioscience Research Reagents), anti-HA (1:1000, Upstate Biotechnology, Lake Placid, NY, USA). The following secondary antibodies were used: Alexa Fluor 488 anti-mouse, Alexa Fluor 568 anti-mouse, Alexa Fluor 488 anti-rabbit and Alexa Fluor 568 anti-rabbit IgG (1:200, Invitrogen), anti-rabbit IgG biotin-conjugated (1:50, Millipore Bioscience Research Reagents), Avidin-Rhodamine (1:50, Vector Laboratories, Burlingame, CA, USA) and Avidin-fluorescein (1:50, Roche, Indianapolis, IN, USA).

### Synapse count and morphometric analysis

To determine the number of synapses per neuron, hippocampal cultures were double-stained with synaptophysin and tubulin antibodies. The number of synaptophysin immunoreactive spots in randomly selected fields was determined using Metamorph Image Analysis Software (Fryer Company Inc., Huntley, IL, USA). To determine synaptic density, cultures were double-stained with synapsin I and MAP 2 antibodies. MAP 2 was used as a dendritic marker (Caceres et al., 1984). The number of synapsin I immunoreactive spots and the neuritic length of MAP 2 immunoreactive processes from randomly selected fields were determined using Metamorph Image Analysis Software. The number of synapsin I immunoreactive spots in each field was divided by the dendritic length in the same field to obtain synaptic density. Sixty fields from three independent experiments were analyzed for each condition. Results were presented as the mean±SEM. Data, obtained blind as to treatment condition, were analyzed using one-way ANOVA followed by Fisher's LSD posthoc test.

### Protein electrophoresis and immunoblotting

Hippocampal neurons and HEK cells were rinsed twice in warmed PBS, scraped into Laemmli buffer, and homogenized in a boiling