RoR2 functions as a noncanonical Wnt receptor that regulates NMDAR-mediated synaptic transmission

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Wnt signaling has a well-established role as a regulator of nervous system development, but its role in the maintenance and regulation of established synapses in the mature brain remains poorly understood. At excitatory glutamatergic synapses, NMDA receptors (NMDARs) have a fundamental role in synaptogenesis, synaptic plasticity, and learning and memory; however, it is not known what controls their number and subunit composition. Here we show that the receptor tyrosine kinase-like orphan receptor 2 (RoR2) functions as a Wnt receptor required to maintain basal NMDAR-mediated synaptic transmission. In addition, RoR2 activation by a noncanonical Wnt ligand activates PKC and JNK and acutely enhances NMDAR synaptic responses. Regulation of a key component of glutamatergic synapses through RoR2 provides a mechanism for Wnt signaling to modulate synaptic transmission, synaptic plasticity, and brain function acutely beyond embryonic development.

NMDA receptors | Wnt signaling | synaptic transmission | RoR2 | glutamate receptors

Wnt ligands are highly conserved secreted glycoproteins responsible for important developmental and homeostatic processes throughout the animal kingdom (1, 2). They play a key role in morphogenesis, patterning, and lineage decision during central and peripheral nervous system development (3). Wnt ligands control gene expression (4), and their dysregulation has been implicated in cancer and major neuropathologies (5–9).

The sustained expression of Wnt ligands and Wnt signaling components in the mature mammalian CNS and their involvement in neuropathologies suggest that these signaling cascades might also play a part in synaptic maintenance and function beyond embryonic development (10, 11). However, because of the pleiotropy and complexity of Wnt signaling, it has been difficult to dissect the components of Wnt signaling present in mature neurons and their role, if any, in the regulation of established synaptic connections and synaptic transmission.

Although most excitatory glutamatergic neurotransmission in the brain is mediated by AMPA-type glutamate receptors [i.e., AMPA receptors (AMPARs)], unique properties allow the NMDA-type glutamate receptors [i.e., NMDA receptors (NMDARs)] to play a critical role in synaptic plasticity, learning and memory, and the establishment and maturation of functional neural circuits (12–14). Despite their importance, it is not known what controls the number and subunit composition of synaptic NMDARs. Dysfunction of NMDARs has been implicated in numerous diseases, including Huntington disease, Parkinson disease, depression, bipolar disorder, and schizophrenia (14, 15), in which a deficit in NMDAR mediated neurotransmission may be central (16). Interestingly, NMDAR-mediated currents can be acutely and specifically up-regulated by Wnt5a, a noncanonical Wnt ligand (17), but little is known regarding the signaling pathway and mechanisms involved in such regulation.

The receptor tyrosine kinase-like orphan receptor 2 (RoR2) is part of a conserved family of tyrosine kinase-like receptors that have been proposed to serve as a receptor for noncanonical Wnt ligands, participating in developmental processes like cell movement and cell polarity (18, 19). Although RoR2 protein has been detected in mammalian neurons (20), its function and signaling pathways are not known. Here we show that RoR2 acts as a receptor for noncanonical Wnt ligands capable of regulating synaptic NMDARs. In hippocampal neurons, activation of RoR2 by noncanonical Wnt ligand Wnt5a activates PKC and JNK, two kinases involved in the regulation of NMDAR currents. In addition, we show that signaling through RoR2 is necessary for the maintenance of basal NMDAR-mediated synaptic transmission and the acute regulation of NMDAR synaptic responses by Wnt5a.

Identification of RoR2 as a Wnt receptor that regulates synaptic NMDARs provides a mechanism for Wnt signaling to control synaptic transmission and synaptic plasticity acutely, and is a critical first step toward understanding the role played by Wnt signaling in the regulation of glutamatergic synaptic function under normal or pathological conditions.

Results

RoR2 Receptor Is Expressed in Hippocampal Neurons of Juvenile Rodents. RoR2 receptors have been implicated in multiple aspects of brain development in *Caenorhabditis elegans* and *Xenopus laevis*, where they have been associated with Wnt5a signaling (18, 19). Although they have been detected in mammalian cultured neurons (20), their function beyond embryonic development, i.e., in the regulation of established synaptic connections, is not known.

In situ hybridization shows that RoR2 mRNA is highly expressed in the cell body of CA1 and CA3 neurons in the mature hippocampus of mice (Fig. 1*A*; Allen Mouse Brain Atlas [21]). RoR2 mRNA is also detected in juvenile rats by using total mRNA isolated from hippocampi and RT-PCR (Fig. 1*B*). Immunostaining of 2-wk-old cultured rat hippocampal neurons confirmed the expression of RoR2 protein and its distribution throughout the cell body and dendrites (Fig. S1*A*). Subcellular fractionation shows that RoR2 is not enriched in a hippocampal synaptosomal preparation (Fig. 1*C* and Fig. S1*B*). Consistent

Significance

NMDA receptors (NMDARs) are key components of excitatory synapses. Here we report that Wnt signaling via activation of tyrosine kinase-like orphan receptor 2 can regulate synaptic NMDARs. Understanding what controls number and subunit composition is central to understand neuropathologies associated with dysfunction of synaptic NMDARs, including Alzheimer's disease and schizophrenia. Our data indicate a previously unidentified role for Wnt signaling in the regulation of established synaptic connections and provides a mechanism for Wnt ligands to modulate basal synaptic transmission, synaptic plasticity, and brain functions acutely.

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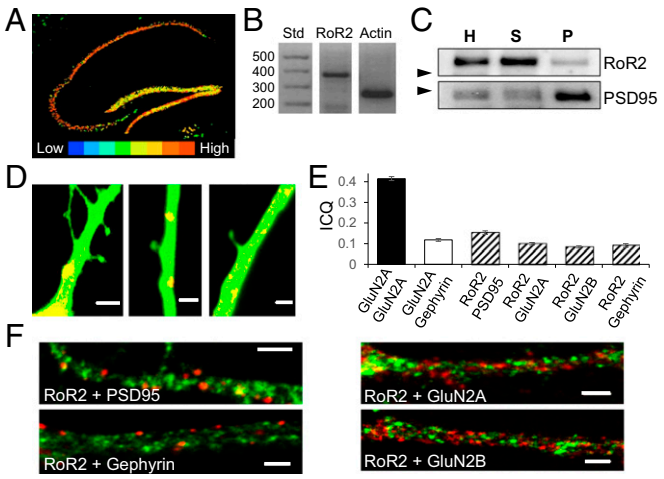

Fig. 1. Expression of RoR2 receptor in juvenile hippocampus. (*A*) In situ hybridization for RoR2 in hippocampus from the Allen Mouse Brain Atlas (mouse.brain-map.org/) (21). Pseudocolor indicates level of expression. (*B*) Reverse transcription reaction from total rat hippocampus mRNA and PCR using specific primers for rat RoR2 or β-actin. (*C*) Representative immunoblot of RoR2 and PSD95 in a synaptosomal preparation (marked as "P"), supernatant (marked as "S"), or total homogenate (marked as "H"). (*D*) Representative images of dendrites from neurons expressing GFP and mCherry-RoR2. (Scale bars: *Left*, 5 μm; *Center* and *Right*, 2 μm.) (*E*) Quantification of colocalization of endogenous RoR2 and synaptic markers. ICQ (22) for RoR2 and PSD95, RoR2 and GluN2A, RoR2 and GluN2B, and RoR2 and gephyrin (stripped bars; $n = 22$, $n = 15$, $n = 8$, and $n = 15$, respectively). The experimental maximum ICQ value is given by the pair mCherry-GluN2A and GFP-GluN2A (black bar; $n = 4$), whereas the minimum is given by the pair GluN2A and gephyrin (white bar; $n = 9$). (*F*) Representative images of dendrites immunostained for RoR2 (green) and PSD95, gephyrin, GluN2A, or GluN2B (synaptic markers in red) in cultured hippocampal neurons. (Scale bar: 2 μm.)

with this, coexpression of GFP and optically tagged RoR2 shows that RoR2 is not present in dendritic spines, the site of glutamatergic synapses (Fig. 1*D*). These data suggest that RoR2 receptors are not targeted to synapses. To confirm this, we quantitatively analyzed colocalization of RoR2 with synaptic markers PSD95 and GluN2 subunits of NMDARs. Immunocytochemistry and intensity correlation analysis (ICA) (22) was used to measure the degree of colocalization of RoR2 receptors with these synaptic markers. ICA calculates the covariation of pixel intensities for a set of proteins across the cell rather than simply pixel location and allows quantification and statistical test level of colocalization or segregation by using an intensity correlation quotient (ICQ; *SI Experimental Procedures*). The maximum experimental ICQ value was obtained by analyzing colocalization of coexpressed mCherry-GluN2A and GFP-GluN2A. The minimum experimental ICQ value was obtained with immunostaining of GluN2A and gephyrin, markers of excitatory and inhibitory synapses, respectively. As shown in Fig. 1 *E* and *F*, RoR2 receptors do not colocalize with PSD95, GluN2A, GluN2B, or gephyrin. The ICQ value for any of these synaptic markers with RoR2 was low, comparable to the minimum experimental ICQ (Fig. 1*E*). These results indicate that RoR2 is distributed along the dendritic tree but it is not targeted to synapses.

RoR2 Overexpression Increases Basal NMDAR-Mediated Synaptic Currents. To investigate whether RoR2 affects glutamatergic synaptic transmission, we measured AMPAR and NMDAR-mediated currents in CA1 neurons overexpressing RoR2. Recombinant rat RoR2 was tagged with mCherry after the predicted signaling peptide and hippocampal slices were transfected by using biolistics (23). A mutant of mCherry-RoR2 lacking the ability to bind Wnt ligands was created by deleting the cysteine rich domain (CRD). This domain, present in the extracellular portion of RoR2, resembles the Wnt binding site of the Frizzled proteins (24) and binds to Wnt5a (19). In addition, a signaling-deficient mCherry-RoR2 was created by deleting most of the intracellular carboxyl terminus (CT) that contains putative signaling domains (25) (Fig. 2*A*). Targeting of these recombinant RoR2 receptors to dendrites was not altered (Fig. S2*A*).

Organotypic hippocampal slices were transfected by using biolistics with mCherry-RoR2 WT or deletion mutants. Transfected CA1 pyramidal cells were identified and synaptic glutamatergic currents recorded in whole-cell configuration. Synaptic responses were evoked by stimulation of the Schaffer collaterals with a bipolar electrode. The amplitude of synaptic responses were compared with responses evoked under the same conditions in a nontransfected adjacent neuron (Fig. 2*B*, paired recordings). AMPAR-mediated responses were recorded at a holding potential of −60 mV. NMDAR-mediated responses

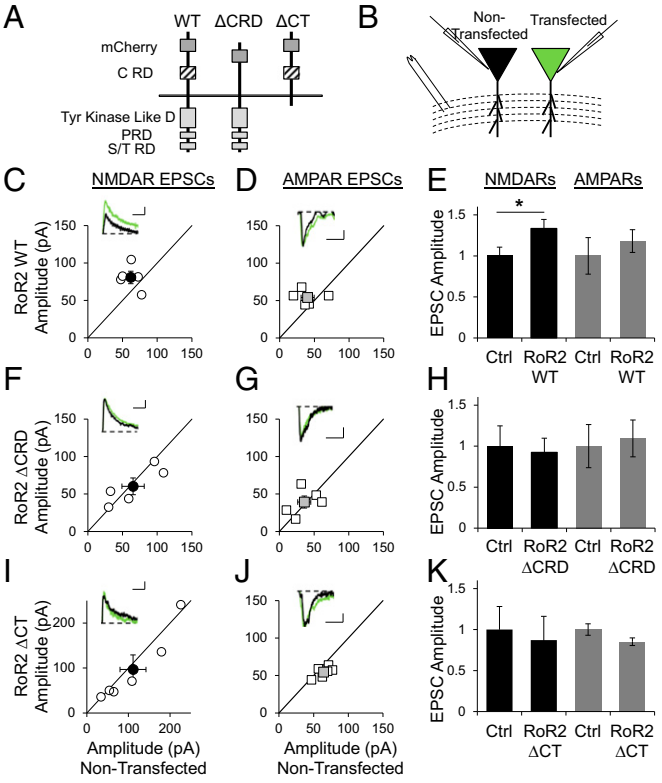

Fig. 2. Overexpression of RoR2 increases basal NMDAR synaptic transmission. (*A*) Schematics of recombinant RoR2 receptors tagged with mCherry used in this study. The CRD and putative intracellular signaling domains Tyr kinase-like domain, proline rich domain (PRD), and a Ser- and Thr-rich domain (S/T RD) are shown. (*B*) Schematic of pairwise recordings in hippocampal slices from a fluorescent transfected CA1 pyramidal neuron and an adjacent nonfluorescent neuron with identical stimulation conditions. (*C*) Peak amplitude of isolated NMDAR synaptic responses (+40 mV; 2 μM NBQX) from neurons overexpressing mCherry-RoR2 (ordinate) and adjacent nontransfected neuron (abscissa). Black circle is average ± SE ($n = 5$ pairs). Here and in all figures, insets are sample traces from paired neurons transfected (green) and nontransfected (black). (*D*) Peak amplitude of AMPAR synaptic responses (−60 mV) from neurons overexpressing mCherry-RoR2 and adjacent nontransfected neuron (□, $n = 5$ pairs). Gray square indicates average ± SE. (*E*) Normalized amplitude of synaptic NMDAR responses (black bars) and AMPAR responses (gray bars) from cells as indicated. (*F–H*) Peak amplitude of pairwise NMDAR responses (*F*; $n = 5$ pairs), AMPAR responses (*G*; $n = 5$ pairs), and quantification of the effect (*H*) for pairs of nontransfected neurons and neurons transfected with mCherry-RoR2 ΔCRD. (*I–K*) Peak amplitude of pairwise NMDAR responses (*I*; $n = 6$ pairs), AMPAR responses (*J*; $n = 6$ pairs), and quantification of the effect (*K*) for pairs of nontransfected neurons and neurons transfected with mCherry-RoR2 ΔCT (*$P < 0.05$).

were recorded at +40 mV in the presence of AMPAR blocker NBQX (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide). Overexpression of recombinant mCherry-RoR2 in CA1 neurons increased the basal NMDAR-mediated synaptic transmission compared with an adjacent nontransfected neuron (Fig. 2 *C* and *E*). No effect was observed in the amplitude of AMPAR-mediated synaptic responses (Fig. 2 *D* and *E*). If the observed increase in basal NMDAR transmission is caused by the expression of recombinant RoR2, expression of signaling-deficient RoR2 mutants should prevent the increase in NMDAR-mediated responses. In effect, expression of ligand binding-deficient RoR2 ΔCRD did not cause any increase in NMDAR- or AMPAR-mediated synaptic responses (Fig. 2 *F–H*). Similarly, overexpression of RoR2 lacking the intracellular signaling domains (RoR2 ΔCT) did not change basal NMDAR- or AMPAR-mediated responses compared with adjacent nontransfected neurons (Fig. 2 *I–K*). These experiments indicate that the increase in NMDAR-mediated transmission is a result of an increase in RoR2 signaling. Thus, these gain-of-function experiments suggest that RoR2 can regulate basal synaptic NMDAR responses.

Knockdown of RoR2 Decreases Basal NMDAR-Mediated Synaptic Transmission. To further test the hypothesis that RoR2 signaling is necessary to maintain normal basal NMDAR function, we generated shRNAs targeting rat RoR2 receptors. The plasmid expressing RoR2 shRNA also expresses GFP, allowing identification of transfected cells. Dissociated cultured hippocampal neurons were transfected with different RoR2 shRNAs to identify the most effective shRNA. RoR2 immunostaining from GFP-expressing neurons was compared with nontransfected cells present in the same field of view (Fig. 3*A*). The most effective shRNAs were shRNA#1 and shRNA#3, two shRNAs targeting different RoR2 sequences. A scrambled shRNA used as control did not affect the level of RoR2 expression (Fig. S2*B*).

To study the effect of knocking down endogenous RoR2 receptors on glutamatergic synaptic transmission, organotypic hippocampal slices were transfected by using biolistics with shRNA#1 or shRNA#3. Transfected CA1 pyramidal cells were identified and synaptic glutamatergic currents recorded in whole-cell configuration. To confirm the efficacy of the shRNA in hippocampal slices, we recovered the cytosol of some transfected and nontransfected CA1 pyramidal neurons and performed RT-PCR by using primers for RoR2, GFP, and actin. As shown in Fig. 3*B*, nontransfected cells contained RoR2 but not GFP, whereas cells expressing RoR2 shRNA contained GFP but not RoR2. Actin was used as a control of the reverse transcriptase reaction. AMPAR- or NMDAR-mediated synaptic responses were compared in a pairwise manner with responses evoked in an adjacent nontransfected neuron. Knockdown of RoR2 with shRNA#1 or shRNA#3 decreased synaptic NMDAR responses compared with adjacent nontransfected neurons (Fig. 3*C* and Fig. S3*A*). This effect is specific for NMDAR-mediated synaptic responses, as AMPAR-mediated responses are not affected (Fig. 3*D* and Fig. S3*B*). Quantification of the effect shows that both shRNAs against RoR2 decrease synaptic NMDAR-responses by approximately 50% with no effect on AMPAR-mediated responses (Fig. 3*E* and Fig. S3*C*). Scrambled shRNA used as a control had no effect on NMDAR- or AMPAR-mediated synaptic responses (Fig. S3 *D–F*). These loss-of-function experiments indicate that presence of RoR2 in the hippocampal slice is necessary to maintain basal NMDAR synaptic transmission.

Rescue of RoR2 Function. To confirm the role of RoR2 in the regulation of synaptic NMDARs, we knocked down endogenous receptors and rescued their function by coexpressing RoR2 shRNA with different forms of recombinant RoR2 fluorescently tagged and resistant to knockdown by the shRNA (RoR2*). CA1 pyramidal neurons cotransfected with RoR2 shRNA and WT mCherry-RoR2* exhibited normal NMDAR-mediated synaptic responses, as expected for a rescued function (Fig. 4 *A* and *C*). This manipulation had no effect in AMPAR-mediated responses (Fig. 4 *B* and *C*).

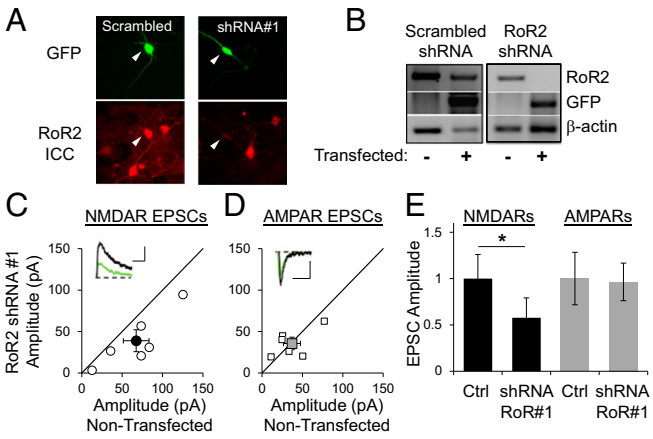

Fig. 3. Knockdown of RoR2 reduces basal NMDAR synaptic transmission. (*A*) Representative images of cultured dissociated neurons (14–20 DIV) transfected with plasmid expressing GFP and RoR2 shRNA or control scrambled shRNA. Cells were immunostained with anti-RoR2 and imaged for GFP (*Top*) or ROR2 immunostaining (*Bottom*). (*B*) Representative single-cell RT-PCR from CA1 pyramidal neurons showing amplification of β-actin as control, GFP, and RoR2 from neurons transfected as indicated. Hippocampal slices were transfected by using biolistics with a plasmid carrying GFP and RoR2 shRNA or scrambled shRNA. (*C*) Peak amplitude of isolated NMDAR synaptic responses (+40 mV; 2 μM NBQX) from neurons expressing RoR2 shRNA#1 and adjacent nontransfected neuron (○, $n = 6$ pairs; ●, average ± SE). (*D*) Peak amplitude of AMPAR synaptic responses (−60 mV) from neurons expressing RoR2 shRNA#1 and adjacent nontransfected neuron (□, $n = 6$ pairs). Gray square indicates average ± SE. (*E*) Normalized amplitude of synaptic NMDAR responses (black bars) and AMPAR responses (gray bars) from cells as indicated.

To establish that RoR2 acts as a receptor of endogenous Wnt ligands and that it is necessary to maintain basal NMDAR transmission, RoR2* ΔCRD, a mutant that cannot bind Wnt ligands (19), was coexpressed with RoR2 shRNA. Coexpression of this mutant with RoR2 shRNA failed to rescue the level of NMDAR-mediated synaptic transmission (Fig. 4 *D* and *F*) and has no effect in AMPAR-mediated synaptic responses (Fig. 4 *E* and *F*). Similarly, the signaling-deficient RoR2 mutant, RoR2* ΔCT, also failed to rescue the normal level of NMDAR synaptic transmission when endogenous RoR2 has been knocked down (Fig. 4 *G* and *I*). AMPAR-mediated synaptic responses were not affected (Fig. 4 *H* and *I*). Together, these experiments establish that RoR2 activation by Wnt ligands and signaling via the intracellular C terminus is necessary to maintain normal levels of NMDAR synaptic transmission.

RoR2 Receptor Is Necessary for Wnt5a Potentiation of NMDAR Currents. Wnt5a, a noncanonical ligand (4), acutely up-regulates the amplitude of NMDAR responses (17), but the mechanism involved in this regulation is not known. We tested whether RoR2 is necessary for this rapid up-regulation of NMDAR currents induced by Wnt5a in hippocampal slices. We recorded isolated excitatory postsynaptic currents mediated by NMDARs in neurons transfected with RoR2 shRNA or in nontransfected neurons. After a 5-min baseline period, we bath-applied conditioned medium containing Wnt5a. In nontransfected neurons or transfected with scrambled shRNA, Wnt5a rapidly potentiated NMDAR currents (Fig. 5*A*, white circles). Wnt5a was not able to potentiate NMDAR currents in neurons in which RoR2 had been knocked down (Fig. 5*A*, black circles, and Fig. S3*G*). Coexpression of RoR2 shRNA with mCherry-tagged RoR2 resistant to degradation (RoR2*) rescued the ability of Wnt5a to potentiate NMDAR currents (Fig. 5*B* and Fig. S3*G*). However, RoR2* mutants lacking the Wnt ligand binding domain (i.e., ΔCRD) or the intracellular carboxyl terminus (i.e., ΔCT) failed to rescue RoR2 function (Fig. 5 *C* and *D*). This set of experiments

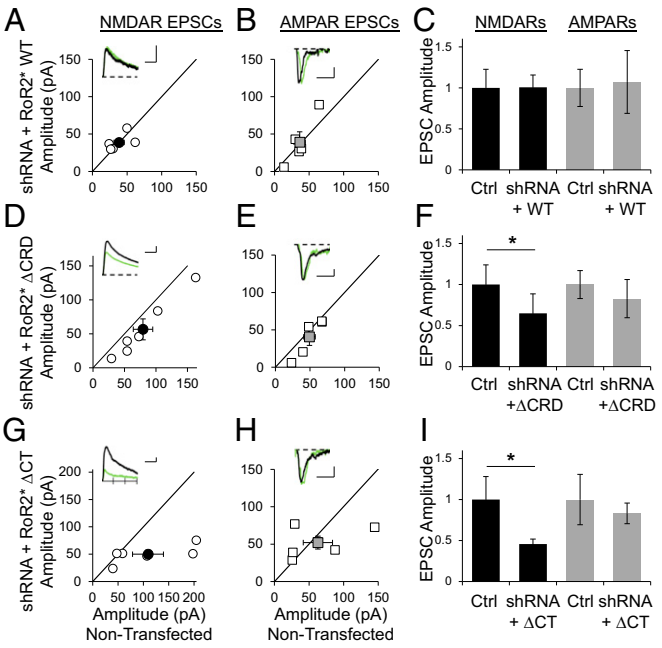

Fig. 4. Rescue of RoR2 function. Effect on basal glutamatergic synaptic responses. (*A*) Peak amplitude of isolated NMDAR synaptic responses (+40 mV; 2 μM NBQX) from neurons coexpressing RoR2 shRNA#1 and WT mCherry-RoR2* (ordinate) and adjacent nontransfected neuron (abscissa; ●, average ± SE, $n = 5$ pairs). (*B*) Peak amplitude of AMPAR synaptic responses (−60 mV) from neurons coexpressing RoR2 shRNA#1 and WT mCherry-RoR2* (ordinate) and adjacent nontransfected neuron (abscissa). Gray square indicates average ± SE ($n = 6$ pairs). (*C*) Normalized amplitude of synaptic NMDAR responses (black bars) and AMPAR responses (gray bars) from control nontransfected cells and transfected as indicated. (*D–F*) Rescue with RoR2*ΔCRD. Peak amplitude of pairwise recordings of NMDAR responses (*D*; $n = 6$ pairs) and AMPAR responses (*E*; $n = 5$ pairs) and quantification of the effect (*F*) for pairs of nontransfected neurons and neurons transfected with RoR2 shRNA and RoR2* lacking the Wnt ligand binding domain. (*G–I*) Rescue with RoR2*ΔCT. Peak amplitude of pairwise recordings of NMDAR responses (*G*; $n = 6$ pairs) and AMPAR responses (*H*; $n = 6$ pairs) and quantification of the effect (*I*) for pairs of nontransfected neurons and neurons transfected with RoR2 shRNA and RoR2* lacking intracellular carboxyl terminus (*$P < 0.05$).

indicates that RoR2 is a receptor for Wnt5a that acutely regulates hippocampal NMDAR synaptic responses.

Noncanonical Wnt Signaling in Hippocampal Neurons: Wnt5a Activates PKC and JNK. To investigate the signaling mechanism involved in the potentiation of NMDAR currents by activation of RoR2, we tested the hypothesis that RoR2 signals via noncanonical pathways upon activation by noncanonical Wnt ligands. We used genetically encoded biosensors of protein kinase activity to address whether Wnt5a is capable of activating PKC and JNK, two kinases involved in the potentiation of NMDARs currents induced by Wnt5a (17). Kinase activity reporters are based on changes in Förster resonance energy transfer (FRET) as a consequence of kinase-specific phosphorylation; PKC activity reporter (CKAR), when phosphorylated by PKC, decreases FRET (26), whereas JNK activity reporter (JNKAR) increases it (27) (Fig. 6 *A* and *B*).

Cultured hippocampal neurons were transfected with CKAR or JNKAR plasmid and live-imaged. A FRET ratio (YFP vs. CFP fluorescence intensity) in the soma as well as in dendrites (Fig. S4 *A* and *B*) was acquired before and after conditioned medium containing Wnt5a, Wnt7a, or control was added to the perfusate. FRET measurements were normalized to the average of baseline. As shown in Fig. 6*C*, Wnt5a decreased the FRET ratio, reaching half-maximal effect 10 min after Wnt5a application, indicating a rapid activation of PKC by Wnt5a. In neurons expressing JNKAR, Wnt5a increased FRET ratio, reaching a stable maximum 30 min after bath application of Wnt5a (half-maximal effect at 17 min), indicating a slower activation of JNK (Fig. 6*D*). Conditioned control medium had no effect on the activity of PKC or JNK kinases. Wnt7a, a canonical ligand, also activated PKC in the soma; however, the increase in PKC activity was small and had a very slow rise time (Fig. 6*C*). JNK activity in the soma also increased marginally following exposure to medium containing Wnt7a, but the effect was not different from that of control medium (Fig. 6*D*). Importantly, Wnt5a was also able to activate PKC and JNK in dendrites (Fig. 6 *E* and *F*), where it could affect NMDAR trafficking. In contrast, Wnt7a failed to activate PKC or JNK in dendrites (Fig. 6 *E* and *F*). The activation of PKC induced by Wnt5a is faster than the activation of JNK in the soma and in dendrites. The activation curves of the kinases in the soma of neurons were fitted with an exponential function with time constants of 10.5 min for PKC and 18.4 min for JNK. Similarly, Wnt5a activation of PKC in dendrites is faster than activation of JNK, with time constants of 32.8 for PKC and 77.2 min for JNK (Fig. 6 *G* and *H* and Fig. S4 *A* and *B*).

RoR2 Mediates Wnt5a Activation of PKC and JNK in Neurons. To test whether RoR2 is necessary for activation of the kinases involved in the potentiation of NMDAR currents, dissociated cultured hippocampal neurons were cotransfected with PKC or JNK activity reporters and shRNA against RoR2. After a baseline was established for the YFP/CFP FRET ratio, Wnt5a was applied to the perfusate. Wnt5a did not activate PKC or JNK when RoR2 had been knocked down (Fig. 7 *A* and *B*). After 30 min without any changes in FRET, PKC or JNK were pharmacologically activated with phorbol-12-myristate-13-acetate (PMA) or anisomycin, respectively, to serve as positive controls. Knockdown of RoR2 also eliminated the small activation of PKC and JNK induced by Wnt7a in the soma of cultured neurons (Fig. S4 *C* and *D*), suggesting that RoR2 could also act as a receptor for Wnt7a although with less efficiency in regard to PKC and JNK activation. Rescue experiments with WT RoR2 restored the ability of Wnt5a to activate PKC and JNK (Fig. 7 *C* and *D*). Also, the small activation of PKC by Wnt7a is restored (Fig. S4*C*). The small JNK activity induced by Wnt7a is not different in neurons in which RoR2 has been knocked down or recued (Fig. S4*D*). These experiments establish that RoR2 mediates the activation of PKC and JNK by Wnt5a in hippocampal neurons.

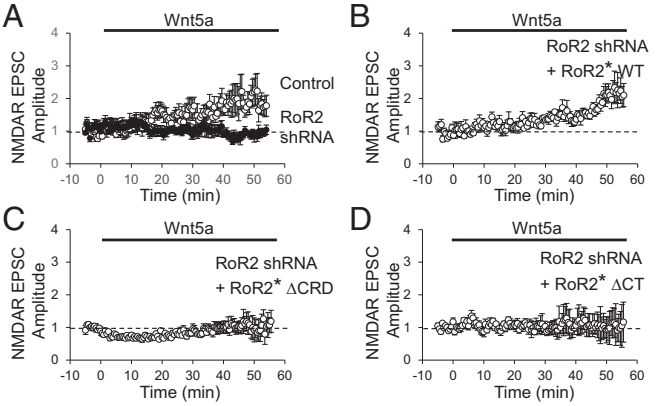

Fig. 5. Wnt5a potentiates NMDAR responses through RoR2. (*A*) Normalized peak amplitude of isolated NMDAR currents recorded at +40 mV in CA1 pyramidal neurons nontransfected and transfected with scrambled shRNA (○; $n = 6$) or transfected with RoR2 shRNA (●; $n = 5$). After a period of baseline, conditioned medium containing Wnt5a was added to the bath. (*B–D*) Normalized peak amplitude of isolated NMDAR currents recorded at +40 mV in CA1 pyramidal neurons cotransfected with RoR2 shRNA and WT RoR2* (*B*; $n = 5$), RoR2* ΔCRD (*C*; $n = 5$), or RoR2* ΔCT (*D*; $n = 5$).

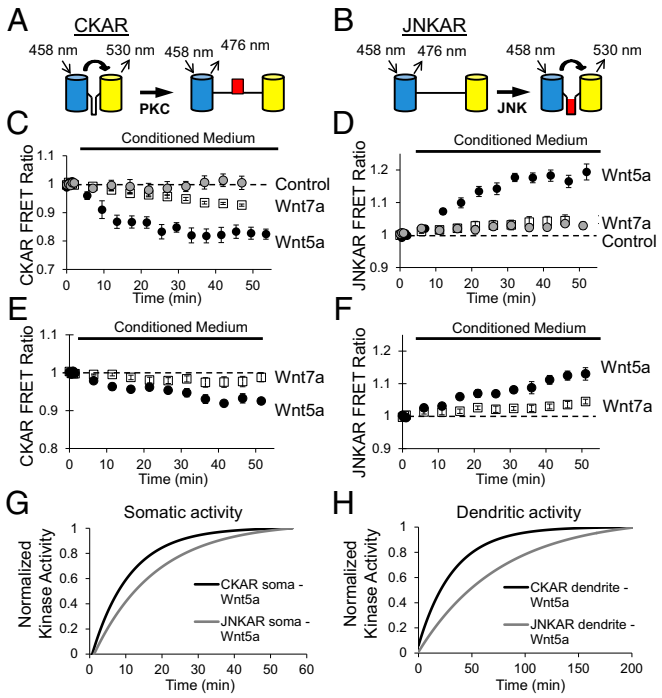

Fig. 6. Activation of PKC and JNK by Wnt5a in hippocampal neurons. (*A* and *B*) Schematics of activity reporters for PKC (CKAR) and JNK (JNKAR) as has been reported (26, 27). Note that activation of PKC decreases FRET between CFP and YFP, whereas activation of JNK increases it. (*C*) Somatic PKC activity measurements. Normalized ratio of YFP/CFP fluorescence intensity from neurons expressing CKAR. A 2-min baseline was acquired before adding conditioned medium from cells expressing control empty vector (gray circles; $n = 7$), Wnt7a (□; $n = 6$), or Wnt5a (●; $n = 9$). (*D*) Somatic JNK activity measurements from cells expressing JNKAR (control, $n = 6$; Wnt7a, $n = 6$; Wnt5a, $n = 9$). (*E* and *F*) Dendritic measurement of PKC activity (*E*) or JNK activity (*F*) from neurons transfected with the corresponding activity reporter and stimulated with Wnt5a (●; $n = 6$) or Wnt7a (□; $n = 6$). (*G*) Wnt5a activation of PKC is faster than activation of JNK. Normalized and estimated activation curves for somatic PKC (black line) and JNK (gray line) according to $K0 + K1 * \exp(-t/\tau)$. CKAR parameters: $K0 = 0.82$; $K1 = 0.19$; $\tau = 10.49$ min. JNKAR parameters: $K0 = 1.20$; $K1 = -0.22$; $\tau = 18.44$ min. (*H*) Normalized and estimated activation curves for dendritic PKC (black line) and JNK (gray line) according to $K0 + K1 * \exp(-t/\tau)$. CKAR parameters: $K0 = 0.91$; $K1 = 0.09$; $\tau = 32.78$ min. JNKAR parameters: $K0 = 1.26$; $K1 = -0.26$; $\tau = 77.18$ min.

Discussion

We have identified the tyrosine kinase-like orphan receptor RoR2 as a receptor for Wnt5a present in hippocampal CA1 neurons that regulates synaptic NMDAR-mediated currents. RoR2 signaling activates PKC and JNK, two kinases involved in the rapid up-regulation of NMDAR currents induced by Wnt5a (17). RoR2 signaling is also necessary to maintain basal level of NMDAR-mediated synaptic transmission. Because of the importance of NMDARs in synaptic function (14, 15), regulation of NMDARs by RoR2 signaling provides a mechanism for Wnt ligands to modulate basal synaptic transmission, synaptic plasticity, and brain functions acutely beyond embryonic development.

Several neuropathologies in adulthood in which dysfunction of NMDARs have been implicated have also been associated with dysregulation of Wnt signaling pathways, including schizophrenia (7, 8), bipolar disorder (28), and Alzheimer's disease (5, 6), suggesting that Wnt signaling cascades might also play a part in synaptic maintenance and function. The requirement of RoR2 signaling to maintain proper levels of NMDAR-mediated synaptic transmission provides a mechanism to better understand some of these neuropathologies in which glutamatergic synaptic transmission has been compromised.

Synaptic NMDARs are more dynamic than originally assumed, with neurons being able to regulate the amount and subunit composition of synaptic NMDARs (29–31). However, the signals and mechanisms controlling the content of synaptic NMDARs are not fully understood. In the present study, by using gain-of-function and loss-of-function experiments, we identify RoR2 signaling as a mechanism that regulates synaptic NMDAR-mediated transmission. The finding that AMPAR-mediated synaptic transmission is not altered serves as a good internal control, indicating that genetic manipulations used here do not compromise the integrity of synaptic transmission.

RoR2 is highly expressed in pyramidal CA1 neurons at an age at which strong synaptogenesis and synaptic plasticity are occurring in the hippocampus. These two processes require proper number and subunit composition of NMDARs (32, 33). Proteomic analysis of postsynaptic densities in humans and rodents shows no presence of RoR2 in PSDs (34); consistent with this, we found that RoR2 is not targeted to synapses. However, dendritic localization of RoR2 receptors and dendritic activation of PKC and JNK place this signaling pathway in the right place to regulate trafficking of NMDARs. Thus, RoR2 is poised to play an important role during hippocampal synaptogenesis and regulation of synaptic function via regulation of synaptic NMDARs.

RoR2 is also responsible for the rapid up-regulation of synaptic NMDARs induced by a noncanonical Wnt ligand, Wnt5a. Importantly, we show that activation of the RoR2 receptor by Wnt5a leads to an increase in the activity of PKC and JNK, two kinases involved in the trafficking of NMDARs toward the surface of neurons and phosphorylation of synaptic scaffolding proteins that anchor NMDARs at synaptic sites (35–37). We found that the time course of activation of PKC is faster than the activation of JNK, suggesting that these kinases could participate in the Wnt5a-induced potentiation of NMDARs via separate mechanisms. Different kinetics of activation induced by Wnt5a also explains why PKC is necessary for a rapid increase in NMDAR-mediated synaptic transmission, whereas JNK seems to potentiate NMDAR currents slowly and in a PKC-independent manner (17). A Wnt ligand considered to be a canonical ligand, Wnt7a, failed to activate PKC and JNK in dendrites but produced a slight increase in the activity of PKC and JNK in the soma of neurons in an RoR2-dependent manner. Because Wnt7a does not potentiate NMDAR currents (17), this level of kinase activation in the soma is not

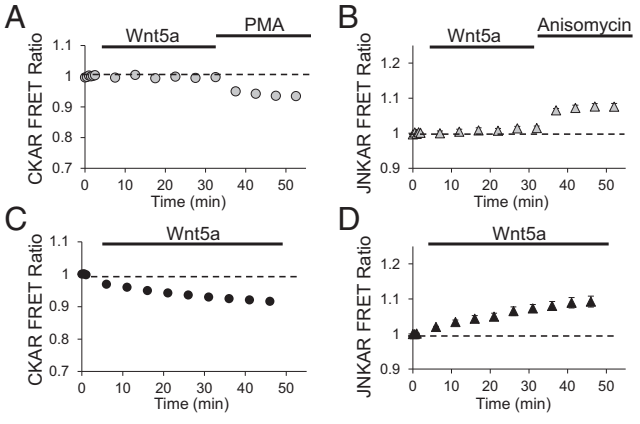

Fig. 7. RoR2 knockdown prevents Wnt5a activation of PKC and JNK. (*A*) PKC activity in cultured hippocampal neurons cotransfected with CKAR and RoR2 shRNA ($n = 8$). After acquisition of baseline, Wnt5a conditioned medium was added to the perfusion bath. After 30 min of no response, PMA (1 μM) was added to the bath. (*B*) JNK activity in cultured hippocampal neurons cotransfected with JNKAR and RoR2 shRNA ($n = 9$) and stimulated with Wnt5a. Anisomycin (8 μM) was added as a positive control. (*C*) PKC activity in neurons cotransfected with RoR2 shRNA and WT RoR2* ($n = 6$) and stimulated with Wnt5a. (*D*) JNK activity in neurons cotransfected with RoR2 shRNA and WT RoR2* ($n = 7$) and stimulated with Wnt5a.

enough to affect NMDAR trafficking, suggesting that the compartmentalized dendritic activity of PKC and JNK is necessary to regulate synaptic NMDARs.

Recent findings argue that the signaling pathways initiated by Wnt ligands may depend on the context of receptors and effectors expressed in a given target cell, rather than on intrinsic properties of the ligands (38). Therefore, it is critical to identify the elements of Wnt signaling present in principal hippocampal neurons that may play a role in regulating glutamatergic synaptic transmission. Identification of RoR2 as a necessary element to regulate NMDAR-mediated synaptic transmission provides a mechanism for Wnt signaling to modulate basal synaptic transmission, synaptic plasticity, and brain functions acutely. Understanding how glutamate receptors are regulated by Wnt ligands has many implications for understanding regulation of processes like learning and memory, synapse formation, and neuropathologies, opening the opportunity to develop truly novel treatments for conditions in which glutamatergic transmission is compromised.

Experimental Procedures

Further details about study methods are provided in *SI Experimental Procedures*.

Optical Measurement of Kinase Activity. CKAR (26) was obtained from Addgene plasmid 14860. JNKAR was obtained from Jin Zhang (Johns Hopkins University, Baltimore, MD) (27). Cultured dissociated neurons [12–17 days in vitro (DIV)] were transfected, and DNA expressed for 36–48 h. Corrected total cell fluorescence (CTCF) for each channel was calculated as integrated density of region of interest (ROI) − (area of ROI * mean background). FRET ratio was calculated as YFP CTCF divided by CFP CTCF. FRET ratios were normalized to the average of baseline.

Knockdown of RoR2. SureSilencing shRNA plasmids from SABiosciences (cat. no, KR55098G) targeting rat RoR2 sequence (NM001107339) were used. RoR2 shRNA#1 targets noncoding sequence GGCTTACGGAGGCATGGTTTTCC. RoR2 shRNA#3 targets coding sequence ATCGCCTGTGCACGCTTCATTGG.

Recombinant RoR2 Receptors. Rat RoR2 cDNA (National Center for Biotechnology Information accession no. NM001107339) containing mCherry after E34 was synthesized (GeneScript) and confirmed by sequencing. Silent mutations were introduced to generate unique restriction sites for further modifications. mCherry RoR2 ΔCRD was generated by removing amino acids R178 through Y313. mCherry RoR2 ΔCT was generated by introducing a stop codon after K466.

Conditioned Medium Containing Wnt Ligands. Wnt5a, Wnt7a, or control empty vector were expressed in HEK-293 cells [DMEM, 10% (vol/vol) FCS, 100 μg/mL streptomycin, and 100 U/mL penicillin] and maintained in Neurobasal medium (no. 21103; GIBCO) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin medium for 60 h. Conditioned media was collected and centrifuged at low speed for 5 min. Twenty milliliters of supernatant were dialyzed (Spectra/Por 4 Dialyzer Tubing, molecular weight cutoff 12–14 KDa; cat. no. 3787D40; Thomas Scientific) for 16–24 h against 2 L of artificial cerebrospinal fluid (ACSF) without calcium. Dialyzed conditioned medium was stored at 4 °C and used within 3–4 d.

ICA. Fluorescence intensity was quantified in matched ROIs for both channels and converted into a data set of pixel intensity pairs. The product of the difference from the mean (PDM) for each intensity pair (Ai − *a*)*(Bi − *b*) was calculated, where *a* and *b* are the means of the distributions with *N* values for Ai (fluorophore A) and Bi (fluorophore B). A ratio of the number of positive PDM values to the total number of pixel pairs was calculated, and 0.5 was subtracted to reflect the degree of dependency. This ICQ ranging from −0.5 to +0.5 is a statistically testable measurement that provides an overall index of whether the intensity values are associated in a random, a dependent, or a segregated manner. Theoretical ICQ values for random or mixed staining/expression is ∼0; those for dependent staining/expression are $0 < \text{ICQ} \leq +0.5$; and those for segregated staining/expression are $0 > \text{ICQ} \geq -0.5$.

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