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Report

LRRTM2 Functions as a Neurexin Ligand in Promoting Excitatory Synapse Formation

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SUMMARY

Recently, leucine-rich repeat transmembrane proteins (LRRTMs) were found to be synaptic cell-adhesion molecules that, when expressed in nonneuronal cells, induce presynaptic differentiation in contacting axons. We now demonstrate that LRRTM2 induces only excitatory synapses, and that it also acts to induce synapses in transfected neurons similarly to neuroligin-1. Using affinity chromatography, we identified α- and β-neurexins as LRRTM2 ligands, again rendering LRRTM2 similar to neuroligin-1. However, whereas neuroligins bind neurexins containing or lacking an insert in splice site #4, LRRTM2 only binds neurexins lacking an insert in splice site #4. Binding of neurexins to LRRTM2 can produce cell-adhesion junctions, consistent with a *trans*-interaction regulated by neurexin alternative splicing, and recombinant neurexin-1β blocks LRRTM2's ability to promote presynaptic differentiation. Thus, our data suggest that two unrelated postsynaptic cell-adhesion molecules, LRRTMs and neuroligins, unexpectedly bind to neurexins as the same presynaptic receptor, but that their binding is subject to distinct regulatory mechanisms.

INTRODUCTION

Synapse assembly, maturation, specification, and maintenance are likely driven by a multitude of *trans*-synaptic cell-adhesion molecules. Multiple synaptic cell-adhesion molecules may contribute to these processes, including, but not limited to, neurexins and neuroligins (Ushkaryov et al., 1992; Ichtchenko et al., 1995), ephrins and Eph receptors (Torres et al., 1998), SynCAMs (Biederer et al., 2002), and netrin G-ligands (NGLs) (Kim et al., 2006). A key technical advance in studying synaptic cell-adhesion molecules was the discovery that expression of such proteins in nonneuronal cells can potently enhance the formation of synapses onto these cells (i.e., induce presynaptic differentiation of axons), when these cells are cocultured with neurons (Scheiffele et al., 2000; Biederer et al., 2002; Graf et al., 2004; Kim et al., 2006). In this assay, referred to as the artificial synapse-formation assay, SynCAMs, neuroligins/neurexins, and NGLs are active (see references cited above). Most recently, a family of neuronal leucine-rich repeat proteins called LRRTMs was also identified as a group of postsynaptic proteins that are active in this assay (Linhoff et al., 2009; Brose, 2009).

LRRTMs comprise a family of four homologous leucine-rich repeat proteins that are selectively expressed in neurons with a differential distribution in brain (Lauren et al., 2003). LRRTM1 is a maternally suppressed gene that is associated paternally with handedness and schizophrenia (Francks et al., 2007; Ludwig et al., 2009). All LRRTMs induce presynaptic differentiation in artificial synapse-formation assays, and LRRTM2 is localized to excitatory synapses (Linhoff et al., 2009). Moreover, deletion of LRRTM1 in mice causes an increase in the immunoreactivity for the vesicular glutamate transporter VGLUT1 (Linhoff et al., 2009), a morphological change similar to that observed in neuroligin-3 R451C knockin mice (Tabuchi et al., 2007). Together, these data indicate that LRRTMs may be postsynaptic cell-adhesion molecules that are similar to neuroligins. However, these data raise important new questions; for example, do LRRTMs also alter synapse numbers in neurons, and more significantly, with which presynaptic molecules do LRRTMs interact?

Here we examined the role of LRRTMs in neurons, focusing on LRRTM2 because of its well-documented localization to synapses (Linhoff et al., 2009). We demonstrate that LRRTM2 selectively induces excitatory synapse formation in the artificial synapse-formation assay, and increases excitatory synapse density in transfected neurons. Moreover, we identify neurexins as the presynaptic receptors for LRRTM2, and demonstrate that neurexin binding to LRRTM2 is tightly regulated by alternative splicing of neurexins at splice site #4 (SS#4). Our data expand the *trans*-synaptic interaction network mediating synaptic cell adhesion, and suggest that neurexins generally nucleate *trans*-synaptic signaling.

RESULTS

LRRTM2 Induces Excitatory Presynaptic Specializations in the Artificial Synapse-Formation Assay

We transfected COS-7 cells with plasmids encoding only mVenus (control), or mVenus-fusion proteins of LRRTM2 or neuroligin-1, and cocultured the transfected COS-7 cells with

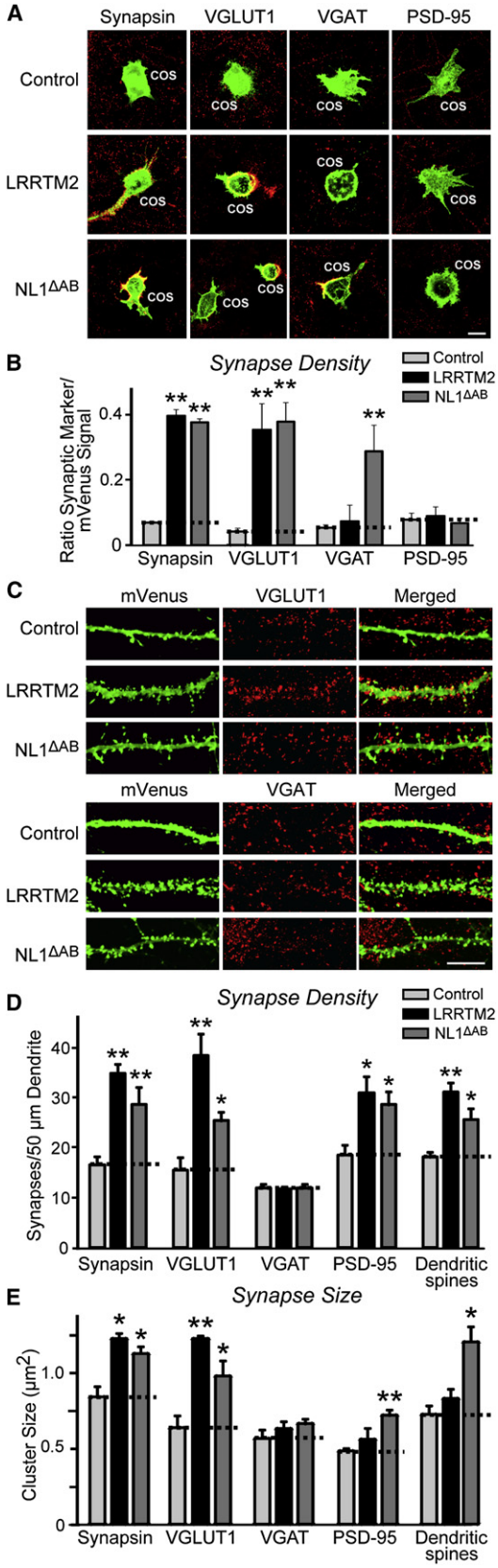

Figure 1. LRRTM2 Expression in COS-7 Cells and in Cultured Hippocampal Neurons Increases Excitatory Synapse Density

(A) LRRTM2 selectively promotes formation of excitatory synapses in the artificial synapse-formation assay. Hippocampal neurons were cocultured for 2 days with COS-7 cells expressing mVenus alone (control), an LRRTM2-mVenus-fusion protein (LRRTM2), or an mVenus-fusion protein of neuroligin-1 lacking inserts in splice sites A and B ($NL1^{\Delta AB}$). Panels show representative immunofluorescence images of the cocultures stained with antibodies to mVenus (green: GFP) and to various presynaptic and postsynaptic markers (red: VGLUT1, vesicular glutamate transporter 1; VGAT, vesicular GABA transporter). Coincident green and red signals are shown in yellow (scale bar = 25 µm; applies to all images). (B) Quantitation of the artificial synapse formation activity of LRRTM2 and neuroligin-1. Experiments as described in (A) were quantified by measuring the ratio of the synaptic marker staining to mVenus fluorescence (for absolute red and green fluorescence values, see Figure S1). (C) Representative images of cultured hippocampal neurons that were transfected at DIV10 with mVenus alone (control), an LRRTM2 mVenus-fusion protein (LRRTM2), or an mVenus-fusion protein of neuroligin-1 lacking inserts in splice sites A and B ($NL1^{\Delta AB}$). Cultures were analyzed at DIV14 by double immunofluorescence with antibodies to mVenus and the synaptic markers described above for (A) (scale bar = 5 µm, applies to all images). (D and E) Effect of LRRTM2 and neuroligin-1 on synapse density (D) and synaptic signal intensity (E), which were quantified with the indicated markers in neurons transfected with mVenus alone (control), the LRRTM2 mVenus-fusion protein (LRRTM2), or the neuroligin-1 mVenus-fusion protein ($NL1^{\Delta AB}$). All data shown are means ± SEMs (n = 3 independent culture experiments). Statistical significance was assessed by comparing the LRRTM2 and neuroligin-1 effects with the control using Student's t test (*$p < 0.05$; **$p < 0.01$).

cultured hippocampal neurons. After 2 days of coculture, samples were fixed, immunolabeled for mVenus and synaptic markers, and analyzed by quantitative fluorescence microscopy (Figures 1A and 1B).

Immunostaining for synapsin confirmed that LRRTM2, similarly to neuroligin-1, induced formation of stable presynaptic terminals onto transfected COS-7 cells (Linhoff et al., 2009). Analysis with antibodies to the vesicular glutamate and GABA transporters (VGLUT1 and VGAT, respectively) demonstrated that LRRTM2 only induced formation of excitatory, VGLUT1-containing synapses on transfected COS-7 cells, whereas neuroligin-1 induced formation of both excitatory and inhibitory synapses (Figures 1A, 1B, and S1A [the latter available online]). Neither LRRTM2 nor neuroligin-1 produced formation of synaptic specializations containing the postsynaptic marker PSD-95.

Neuronal Overexpression of LRRTM2 Increases Excitatory Synapse Numbers

We next examined whether the effect of LRRTM2 can also affect synapse density in neurons. We transfected cultured hippocampal neurons at 10 days in vitro (DIV10) with plasmids encoding only mVenus (control), or mVenus-fusion proteins of LRRTM2 or neuroligin-1, and fixed and immunostained the neurons at DIV14. Using image analysis, we quantified the density and signal intensity of immunoreactive synaptic puncta identified with antibodies for various presynaptic and postsynaptic markers and for mVenus (Figures 1C–1E). LRRTM2 potently increased the density of excitatory, but not inhibitory, synapses on transfected neurons, as measured with both presynaptic and postsynaptic marker proteins. In this assay, LRRTM2 thus again acted similarly to neuroligin-1, but was more effective (Figures

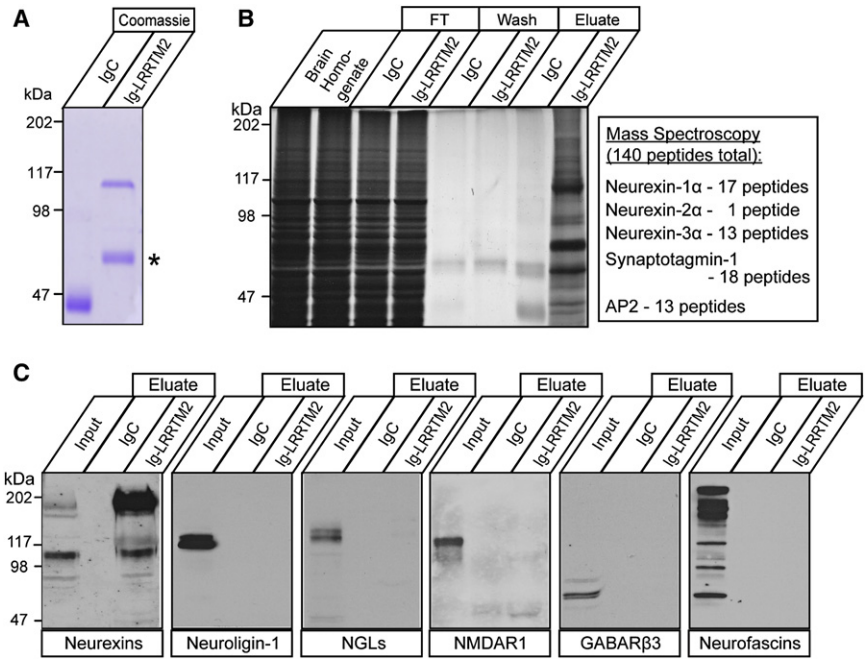

Figure 2. Affinity Purification of Neurexins on Immobilized LRRTM2

(A) Coomassie-stained gel of recombinant Ig-control (IgC) and Ig-LRRTM2-fusion proteins used for affinity chromatography and pull-down experiments. In the Ig-LRRTM2 lane, the lower band (asterisk) is likely a breakdown product of the full-length Ig-LRRTM2-fusion protein. (B) Identification of neurexins as LRRTM2-receptors. Proteins solubilized with Triton X-100 from total rat brain homogenate (Brain Homogenate) were passed through a column containing immobilized IgC or Ig-LRRTM2. After collection of the flow-through (FT), the column was washed extensively with homogenization buffer (Wash), and eluted with 0.5 M NaCl (not shown) and 1.0 M NaCl (Eluate). Fractions were analyzed by SDS-PAGE and silver staining as shown, and eluted proteins above 80 kDa were subjected to mass spectrometry, with identification of 140 peptides as indicated on the right. (C) Immunoblotting analysis of solubilized rat brain membrane proteins (Input; 5% of total) subjected to pull-down experiments with IgC or Ig-LRRTM2-fusion protein. Equivalent amounts of bound proteins were analyzed with the antibodies indicated below the panels (GABARβ3, β3 subunit of $GABA_A$-receptors; NGLs, netrin-G ligands; NMDAR1, NMDA receptor subunit 1).

1C–1E). Moreover, LRRTM2 strongly increased the signal of presynaptic markers per punctum on the transfected neurons, also similarly to neuroligin-1. However, unlike neuroligin-1, LRRTM2 had no significant effect on the postsynaptic signal (Figures 1C–1E).

Neurexins Are Candidate Presynaptic Ligands for LRRTM2

To search for presynaptic ligands of postsynaptic LRRTM2, we produced recombinant LRRTM2 composed of the entire extracellular sequence of LRRTM2 fused to the Fc-domain of human IG (Ig-LRRTM2), analogous to previously generated neurexin-fusion proteins (Ushkaryov et al., 1994). As a control, we used IgC that is composed of the first 18 residues of mature neurexin-1α fused to the Fc-domain of human IG. Ig-fusion proteins were produced in transfected HEK293T cells, and purified on protein A-Sepharose (Figure 2A). We then performed affinity chromatography experiments with solubilized rat brains on the immobilized Ig-fusion proteins (Figure 2B).

Silver staining of SDS polyacrylamide gels loaded with the input, wash, and eluate fractions from affinity chromatography experiments suggested that multiple bands are purified on immobilized LRRTM2. We performed mass spectrometry analyses of all proteins larger than Ig-LRRTM2 in the silver-stained gels. Of 140 identified peptides, 31 peptides were derived from neurexins, with all three α-neurexins represented, and 18 peptides were from synaptotagmin-1, which binds to neurexins (Figure 2B; Hata et al., 1993). Besides neurexins, no other cell-surface proteins were identified in the LRRTM2 affinity-purified fractions, suggesting that neurexins are the most abundant and/or the most tightly bound extracellular interaction partners of LRRTM2.

To confirm that LRRTM2 indeed pulls down neurexins present in detergent-solubilized membrane fractions, we used immunoblotting to analyze which proteins were captured by immobilized LRRTM2 (Figure 2C). We observed a high degree of enrichment of α- and β-neurexins in the LRRTM2-bound fraction, but not of any other cell-surface protein tested, confirming the mass spectroscopy results.

LRRTM2 Directly Binds α- and β-Neurexins in Cell Surface Labeling Assays

To test whether neurexins can directly bind to LRRTM2 on the surface of a cell, we bound various recombinant Ig-fusion proteins of neurexins to HEK293T cells that express full-length LRRTM2-mVenus. We fixed the cells without detergent, and measured surface-bound Ig-fusion proteins by indirect immunofluorescence (Figure 3A). As a negative control, we used IgC, and as a positive control, we employed cells transfected with two different neuroligin-1 splice variants. We found that both neurexin-1α and -1β avidly bound to LRRTM2 in this assay, but only when the neurexins lacked an insert in SS#4. In contrast (but as reported previously; see Boucard et al., 2005), neurexin-1α containing or lacking an insert in SS#4 did not bind to neuroligin-1 containing an insert in splice sites A and B, but did bind to neuroligin-1 lacking an insert in splice sites A and B. Neurexin-1β, similarly to neurexin-1α, also bound to LRRTM2 dependent on SS#4 of neurexin-1β, whereas its binding to neuroligin-1 was independent of neurexin-1β alternative splicing (Figure 3A). Thus, binding of neurexin-1α and -1β to LRRTM2 and to neuroligin-1 is differentially controlled by alternative splicing at SS#4 of neurexins, suggesting that neurexin binding to LRRTM2 and neuroligins operates via distinct but related mechanisms. This conclusion is reinforced by the finding that

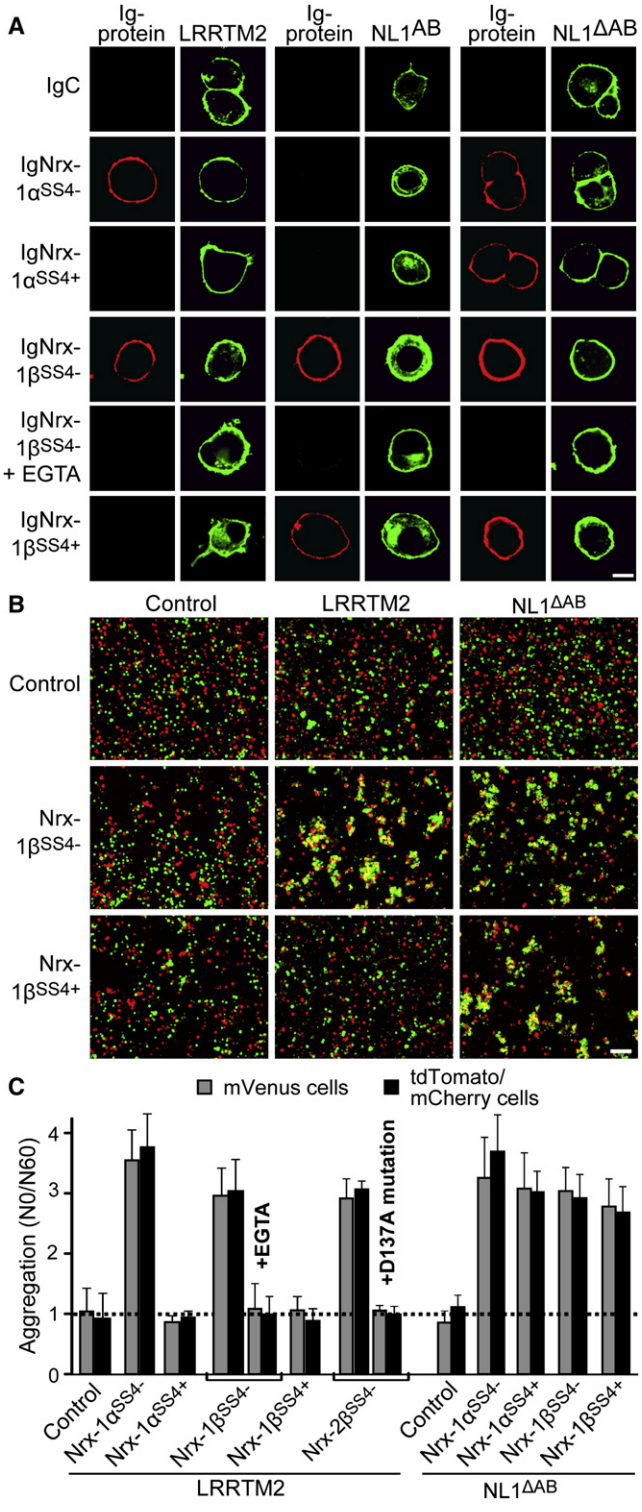

Figure 3. Analysis of LRRTM2/Neurexin Interaction with Cell-Surface Binding and Cell-Adhesion Assays

(A) Surface-binding assays. HEK293T cells expressing mVenus-fusion proteins of LRRTM2, or of two different splice variants of neuroligin-1 ($NL1^{AB}$, which contains an insert in splice sites A and B, and $NL1^{\Delta AB}$, which lacks in insert in these sites), were incubated with control Ig-fusion protein (IgC) or various neurexin Ig-fusion proteins ($IgNrx1\alpha^{SS4-}$, $IgNrx1\alpha^{SS4+}$, $IgNrx1\beta^{SS4-}$, and $IgNrx1\beta^{SS4+}$ = Ig-fusion proteins of neurexin-1α and -1β, respectively, lacking or containing an insert in splice site #4, respectively). Cells were analyzed by immunofluorescence imaging for the Ig-fusion proteins (red) and mVenus (green). All binding reactions were carried out in 2 mM $CaCl_2$, except for the reaction marked by "EGTA," which was performed in 10 mM EGTA (scale bar = 4 μm, applies to all images). For further controls, see Figure S2. (B and C) Representative images (B) and quantitation (C) of cell-adhesion assays. HEK293T cells expressing mVenus (either alone [control] or as an LRRTM2- or $NL1^{\Delta AB}$-fusion protein) were mixed with HEK293T cells expressing tdTomato (Control) or mCherry fused with various neurexins ($Nrx1\alpha^{SS4-}$, $Nrx1\alpha^{SS4+}$, $Nrx1\beta^{SS4-}$, and $Nrx1\beta^{SS4+}$ = neurexin-1α and -1β, respectively, lacking or containing an insert in splice site #4 as indicated; $Nrx2\beta^{SS4}$ = neurexin-2β lacking an insert in splice site #4; $Nrx2\beta^{SS4}$ was also examined with a point mutation [D137A] that blocks neuroligin binding [Reissner et al., 2008]). Cells were imaged, and free cell numbers were counted immediately after respective cell populations had been mixed (T_0), and again after 60 min (T_{60}; shown in representative images in B; scale bar = 100 μm, applies to all images). For additional representative images, see Figure S3. Cell aggregation in (C) is calculated by dividing T_0 by T_{60} (data shown are means ± SEMs; n = 3 independent experiments; gray and black bars show quantitations for mVenus and tdTomato/mCherry-expressing cells, respectively; for more quantitations and controls, see Figure S4).

binding of both neurexin ligands is reversed by EGTA, i.e., is Ca^{2+} dependent (Figure 3A).

LRRTM2 Binding to Neurexins Mediates Cell Adhesion

To investigate whether binding of LRRTM2 to neurexins is a *trans*-interaction, i.e., capable of promoting intercellular cell adhesion (as expected for an interaction between a postsynaptic cell-adhesion molecule and presynaptic neurexins), we examined the ability of surface-expressed LRRTM2 to mediate cell adhesion with cells expressing neurexins. We transfected HEK293T cells with (1) mVenus or tdTomato alone; (2) mVenus-fusion proteins of LRRTM2; (3) mVenus-fusion protein of neuroligin-1, (4) mCherry-fusion proteins of various neurexins; and (5) tdTomato cotransfected with untagged LRRTM2. One day after transfection, cells were dissociated, and mVenus-expressing and tdTomato- or mCherry-expressing cells were mixed. Cells were imaged immediately after mixing and after a 60 min incubation at room temperature with mild agitation, and free cells were counted at each time point to quantify cell adhesion (Figures 3B and 3C).

Because at least in some instances, leucine-rich repeat proteins mediate homophilic cell adhesion (e.g., see the role of connectin in *Drosophila* synapse formation; Nose et al., 1992), we first examined whether LRRTM2 mediates homophilic cell adhesion by mixing red (tdTomato) and green (mVenus) LRRTM2-expressing cells. However, we observed no cell adhesion (Figure S3A). We next examined whether LRRTM2 binding to neurexins can mediate cell adhesion. Indeed, cells expressing LRRTM2 formed large aggregates with cells expressing neurexin-1α or -1β, provided that the neurexins lacked an insert in SS#4 ($Nrx1\alpha^{SS4-}$ and $Nrx1\beta^{SS4-}$, but not $Nrx1\alpha^{SS4+}$ or $Nrx1\beta^{SS4+}$; Figures 3B and 3C). All three β-neurexins bound with the same splice-site dependence (Figures S3B and S3C). This splice-site dependence was different from that observed with neuroligin-1, where cells expressing neuroligin-1 lacking

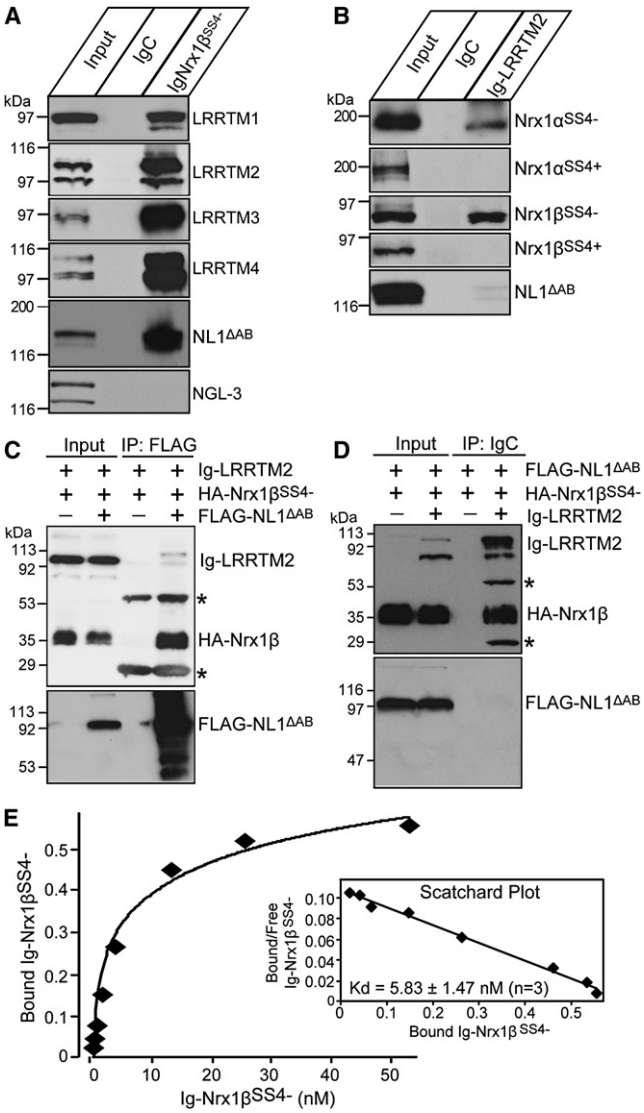

Figure 4. Exclusive Binding of Neurexin-1β to Either LRRTM2 or Neuroligin-1, and Estimation of the LRRTM2-Neurexin Interaction Affinity

(A) Immunoblot analysis of pull-downs of LRRTM1–4 expressed as EGFP- or mVenus-fusion proteins in HEK293T cells. Pull-downs were performed with immobilized Ig-control (IgC) and Ig-neurexin-fusion proteins (IgNrx1β^{SS4-}, lacking an insert in splice site #4), using neuroligin-1 (NL1$^{\Delta AB}$) as a positive control, and netrin G-ligand 3 (NGL-3) as a negative control (Input = 5% of total). Numbers on the left in this and subsequent panels indicate sizes of molecular weight markers. (B) Same as (A), except that pull-downs were performed with IgC and Ig-LRRTM2-fusion proteins (Ig-LRRTM2), and that we analyzed binding of neurexin-1α and -1β lacking or containing an insert in splice site #4 (Nrx1α^{SS4-}, Nrx1α^{SS4+}, Nrx1β^{SS4-}, and Nrx1β^{SS4+}, respectively; all expressed as mCherry-fusion proteins), using neuroligin-1 (NL1$^{\Delta AB}$, expressed as an mVenus-fusion protein) as a positive control. (C) Neurexin-1β bound to neuroligin-1 cannot simultaneously bind to LRRTM2. Purified Ig-LRRTM2, HA-tagged neurexin-1β (HA-Nrx1β^{SS4-}), and FLAG-tagged neuroligin-1 (FLAG-NL1$^{\Delta AB}$; 3 μg of each protein) were mixed and immunoprecipitated with FLAG antibodies. Input (5% of total) and bound proteins were analyzed by immunoblotting as indicated. Asterisks (*) indicate IgG heavy or light chains. (D) Neurexin-1β bound to LRRTM2 cannot simultaneously bind to neuroligin-1. Purified Ig-LRRTM2, HA-Nrx1β^{SS4-}, and FLAG-NL1$^{\Delta AB}$ (3 μg of each protein) were mixed and immunoprecipitated with protein A. Input (5% of total) and bound proteins were analyzed by immunoblotting as indicated. Asterisks (*) indicate IgG heavy and light chains. (E) Saturation binding of Ig-neurexin-fusion protein (Nrx1β^{SS4-}) to LRRTM2 expressed in HEK293T cells. Different concentrations of Nrx1β^{SS4-} were incubated with HEK293T cells transfected with mVenus-fusion protein of LRRTM2 or GW1 vector alone (control), and the amount of bound Nrx1β^{SS4-} was measured using an HRP-labeled secondary antibody. The control signal observed in GW1 vector-transfected HEK293T cells was subtracted to calculate net binding signals. The inset shows a Scatchard plot analyzed by linear regression of the data, with the calculated K_d as determined by three independent experiments.

an insert in the neuroligin-splice sites A and B (NL1$^{\Delta AB}$) formed aggregates with all neurexins tested, consistent with the binding data shown in Figure 3A.

However, similarly to neuroligin-1, LRRTM2-mediated cell adhesion to neurexin-containing cells was strictly dependent on Ca^{2+} binding to neurexins. Binding was abolished by EGTA, and was blocked by a single amino-acid substitution in neurexin-2β that abolishes Ca^{2+} binding to the neurexin LNS domain, and also abolishes neuroligin-1-mediated cell adhesion (Figures 3B, 3C, S3B, and S4). No cell adhesion in negative controls was observed (Figures 3B and S4). Taken together, these data demonstrate that LRRTM2 forms an intercellular junction via a *trans*-interaction with α- and β-neurexins in a manner that resembles that of neuroligin-1, but differs from neuroligin-1 in its distinct regulation of the neurexins by alternative splicing.

LRRTM2 and Neuroligin-1 Cannot Bind Simultaneously to Neurexin-1β

To corroborate the surface-binding and cell-adhesion assays with an independent approach, and to additionally test whether neurexins also bind to other LRRTM isoforms, we performed pull-down experiments using the immobilized Ig-fusion protein of neurexin-1β lacking an insert in SS#4 (IgNrx1β^{SS4-}; Figure 4A). We found that immobilized Ig-neurexin-1β effectively captured all LRRTM isoforms (LRRTM1–4) and neuroligin-1 expressed in HEK293T cells. However, Ig-neurexin-1β did not bind to NGL-3, and IgC used as a negative control was unable to bind to either LRRTMs or neuroligin-1.

We next reversed the assay, and employed the Ig-fusion protein of LRRTM2 described above (see Figure 2) to pull down neurexins expressed as mCherry-fusion proteins in HEK293T cells. Strikingly, LRRTM2 pulled down only neurexins lacking an insert in SS#4, and exhibited no activity toward neuroligin-1, confirming the binding specificity observed in the cell-surface binding and cell-adhesion assays (Figure 4B).

Neurexin binding by both neuroligin-1 and LRRTM2 depends on Ca^{2+} binding to neurexins, and both binding reactions are regulated, although differentially, by SS#4 of neurexins. These observations indicate that despite their lack of homology, LRRTM2 and neuroligin-1 may bind to overlapping sites on the neurexin LNS domain. To test this hypothesis, we investigated whether LRRTM2 and neuroligin-1 simultaneously bind to neurexins. We produced recombinant Ig-LRRTM2, FLAG-tagged neuroligin-1 lacking an insert in splice sites A and B (FLAG-NL1$^{\Delta AB}$), and HA-tagged neurexin-1β lacking an insert in SS#4

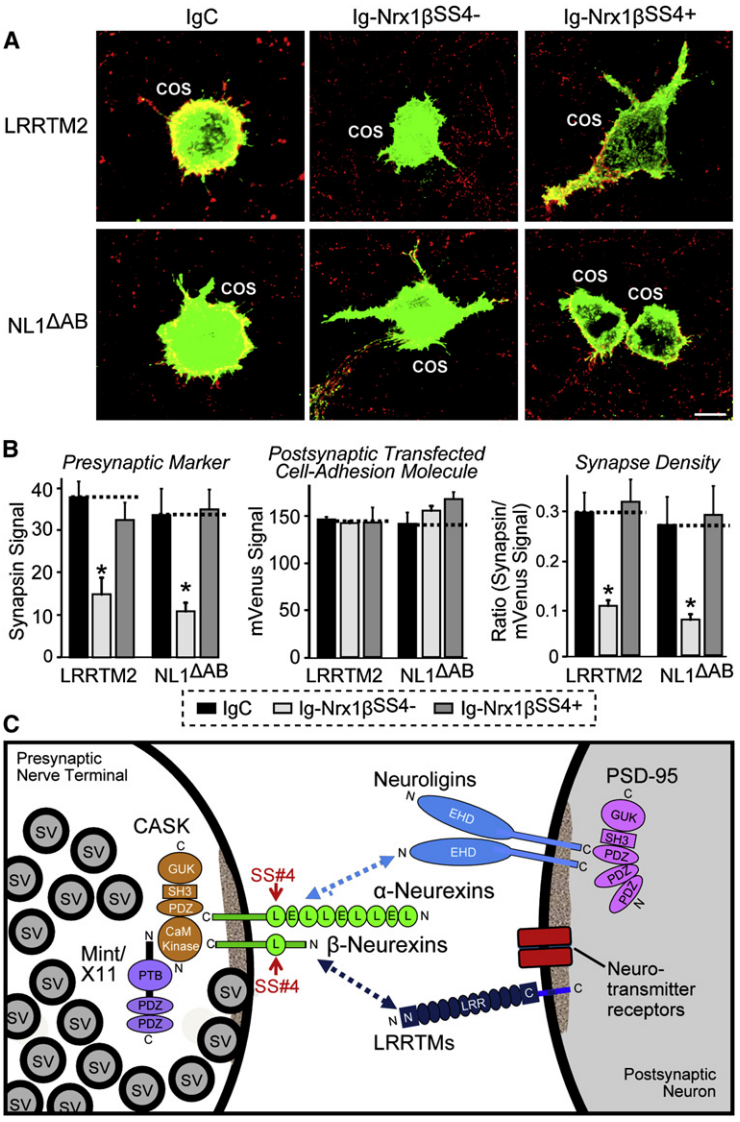

Figure 5. Soluble Ig-Neurexin Impairs Synaptogenic Activity of LRRTM2: Model of LRRTM2 Action

(A and B) Representative images (A) and quantitation (B) of artificial synapse-formation assays to measure the effect of Ig-neurexin-fusion protein lacking (Nrx1β^{SS4-}) or containing (Nrx1β^{SS4+}) an insert in splice site #4 on the synaptogenic activities of LRRTM2 and neuroligin-1. Cultured hippocampal neurons (DIV7) were cocultured for 12 hr with COS-7 cells expressing mVenus-fusion proteins of LRRTM2 or neuroligin-1 lacking inserts in splice sites A and B (NL1$^{\Delta AB}$). Cocultures were performed in the presence of 50 μg/ml of IgC (control), IgNrx1β^{SS4-}, or IgNrx1β^{SS4+}. Panels in (A) show representative immunofluorescence images of the cocultures stained with antibodies to mVenus (green, GFP) and synapsin (red; coincident signals, yellow; scale bar = 25 μm, applies to all images). In (B), the immunofluorescence intensities for synapsin and for mVenus were measured over the same transfected COS-7 cells (left and middle bar graphs), and the ratios of synapsin to mVenus fluorescence signal were determined (right bar graph). Dashed lines correspond to the IgC-treated values as the baseline. Data shown are means ± SEMs (n = 3 independent experiments, *p < 0.05). (C) Model for the *trans*-synaptic interactions of neurexins with neuroligins and LRRTM2. Both bind to neurexins via the 6th LNS domain that is shared by α- and β-neurexins, but exhibit distinct specificities: neuroligins bind to neurexins in a manner that is modulated by alternative splicing at site #4 without being absolutely dependent on this splice site, whereas LRRTM2 binds to neurexins only if splice site #4 lacks an insert.

(HA-Nrx1β^{SS4-}). We then mixed these proteins in different combinations, and tested whether pull-downs of neuroligin-1 or LRRTM2 would capture not only neurexin-1β, but also LRRTM2 or neuroligin-1, respectively (Figures 4C and 4D).

Unequivocally, neurexin-1β, but not LRRTM2, was brought down with neuroligin-1. Similarly, only neurexin-1β, but not neuroligin-1, was brought down with LRRTM2 (Figures 4C and 4D). These data demonstrate that neuroligin-1 and LRRTM2 cannot simultaneously bind to neurexin-1β.

Finally, we estimated the binding affinity for the interaction of LRRTM2 and neurexin-1β. LRRTM2-mVenus-expressing and control HEK293T cells were incubated with increasing amounts of Ig-neurexin-1β-fusion protein lacking an insert in SS#4, and proteins bound to the cell surface were measured with an HRP-tagged secondary antibody. After subtraction of nonspecific binding, we calculated a nanomolar K_d by Scatchard analysis (5.83 ± 1.47 nM; Figure 4E). This result indicates that LRRTM2 binds to neurexins with high affinity, although it should be noted that the dimeric neurexin ligands used in these experiments produce an increased apparent binding affinity.

Soluble Neurexin-1β Impairs the Synaptogenic Activity of LRRTM2

Since neurexins directly interact with LRRTM2 (Figures 2, 3, and 4), the question arises of whether this interaction is essential for the synaptogenic activity of LRRTM2 (Figure 1). As a first test of this question, we examined whether Ig-neurexin-1β lacking or containing an insert in SS#4 (IgNrx1β^{SS4-} and IgNrx1β^{SS4+}) can block the function of LRRTM2 in the artificial synapse formation assay (Figures 5A and 5B). In these experiments, we used IgC as a negative control and neuroligin-1 as a positive control, and restricted the coculture time to 12 hr to avoid cellular uptake and degradation of the Ig-fusion proteins (Chubykin et al., 2005).

IgNrx1β^{SS4-}, but not IgNrx1β^{SS4+}, specifically reduced the synaptogenic activity of LRRTM2 (Figures 5A and 5B), suggesting that neurexins are indeed presynaptic receptors for LRRTM2 in the artificial synapse formation assay, similar to neuroligin (Ko et al., 2009). Interestingly, the synaptogenic activity of neuroligin-1 was also specifically reduced by IgNrx1β^{SS4-}, but not by IgNrx1β^{SS4+}, although neuroligin-1 (NL1$^{\Delta AB}$) binds to both neurexin isoforms in vitro (Figure 3), possibly because of the differences in neuroligin-1 binding affinities of the two different neurexin splice variants (Boucard et al., 2005), or because of the dependence of the artificial synapse-formation assay on α-neurexin binding by neuroligin-1 (Ko et al., 2009).

DISCUSSION

Synaptic cell adhesion not only mediates the initial establishment of synapses, but also directs synapse specification, controls synapse maintenance, and regulates synapses during long-term synaptic plasticity. Moreover, the recent discovery of multiple synaptic cell-adhesion molecules as candidate genes for cognitive diseases such as autism, schizophrenia, and addiction has moved synaptic cell adhesion into the center of attention (reviewed in Südhof, 2008). In particular, neurexin-1 has been repeatedly linked to autism and schizophrenia (Kim et al., 2008; Kirov et al., 2009; Rujescu et al., 2009), LRRTM1 was linked to schizophrenia (Francks et al., 2007; Ludwig et al., 2009), and neurexin-3 has been associated with reward pathways and drug addiction (Bierut et al., 2007). Thus, the recent findings that LRRTMs are candidate synaptic cell-adhesion molecules that are potent effectors in the artificial synapse-formation assay (Linhoff et al., 2009) were of great interest because they suggested that LRRTMs may define a novel *trans*-synaptic cell-adhesion pathway that may contribute to cognitive diseases. However, these results also raised important questions, namely whether the artificial synapse-formation activity of LRRTMs truly reflects a role in synapses formed between neurons, whether this role applies to all types or specific subtypes of synapses, and whether LRRTMs act as homophilic cell-adhesion molecules analogous to the leucine-rich repeat protein connectin (Nose et al., 1992), or function by binding to an as yet unidentified presynaptic ligand.

In the present study, we have provided initial answers to these questions by focusing on LRRTM2, the most abundant LRRTM isoform. We show that LRRTM2 increases the abundance of excitatory, but not inhibitory, synapses, not only in the artificial synapse-formation assay, but also in transfected neurons. Moreover, we demonstrate that LRRTM2 is not a homophilic cell-adhesion molecule, but instead binds to presynaptic neurexins in a tight interaction that is regulated by alternative splicing of neurexins at SS#4. Thus, our data unexpectedly show that neurexins act by binding to two different downstream ligands, neuroligins and LRRTMs, in a mutually exclusive manner, and that their binding to these ligands is differentially regulated. As illustrated in the model (Figure 5C), these findings place neurexins at the core of two separate *trans*-synaptic cell-adhesion pathways, and indicate that the involvement of LRRTM1 and neurexin-1 in schizophrenia may delineate a common mechanism.

As synaptic cell-adhesion molecules, LRRTMs could be involved in one or several steps during synapse formation, from their initial establishment to their maturation and remodeling. The assays used in the present study—the artificial synapse-formation and neuronal transfection assays—do not allow conclusions about the steps in which a protein functions. For example, in the artificial synapse-formation assay, even control nonneuronal cells form transient synapses with cocultured neurons; thus, if a molecule stabilizes or validates synapses, it could in this assay stabilize synapses that are initially established by an independent mechanism. In vivo, neuroligins appear to be more important for synapse specification, function, and plasticity than for the initial establishment of synapses (Varoqueaux et al., 2006; Chubykin et al., 2007), and neurexins have a demonstrated role in synapse specification and function, but not synapse establishment (Missler et al., 2003; Kattenstroth et al., 2004; Zhang et al., 2005). However, these findings do not necessarily mean that LRRTMs do not function in the establishment of initial synapses, and even neurexins might do so, because no complete neurexin deletion has yet been analyzed. Moreover, we do not know whether LRRTMs also bind to other presynaptic ligands, which is a distinct possibility because neuroligins also appear to interact with other presynaptic ligands besides neurexins (Ko et al., 2009). Again, these are issues that will have to be addressed in future studies.

EXPERIMENTAL PROCEDURES

Artificial Synapse-Formation Assays

Artificial synapse-formation assays were performed with COS-7 cells and cocultured hippocampal neurons as described (Ko et al., 2009), and were analyzed after 48 hr.

Affinity Chromatography

Affinity chromatography experiments were performed with rat brain proteins solubilized with 1% Triton X-100, using immobilized LRRTM2-Ig or IgC-fusion proteins as a matrix (Sugita et al., 2001; Boucard et al., 2005). Proteins bound to LRRTM2 affinity matrix were identified by mass spectrometry (Stanford University Mass Spectrometry Facility), and identifications were confirmed by immunoblotting (Figure 2).

Cell-Surface Binding Assays

Cell-surface binding assays were performed with HEK293T cells as described (Boucard et al., 2005).

Cell Aggregation Assays

Cell aggregation assays were performed with HEK293T cells essentially as described (Nguyen and Südhof, 1997). HEK293T cells expressing red or green fluorescent proteins were mixed 24 hr after transfection, and incubated at 4°C under gentle agitation. Cell and aggregate numbers were counted immediately after mixing (N_0), and after 60 min incubation (N_{60}). Aggregation is calculated as N_0/N_{60}.

Primary Neuronal Culture, Transfections, Immunocytochemistry, Image Acquisition, and Analyses

Cultured hippocampal neurons were transfected with LRRTM2-mVenus, $NL1^{\Delta AB}$-mVenus, or mVenus at DIV10, and immunostained at DIV14 by the indicated antibodies as described (Ko et al., 2009). Images of randomly chosen transfected neurons were acquired using a confocal microscope (LSM510, Zeiss or TCS2, Leica) with constant image settings. Z-stacked images were converted to maximal projection, and were analyzed using MetaMorph Software with area size and density of spines and presynaptic terminals per 50 μm of dendrite.

Constructs, Antibodies, and Miscellaneous Procedures

All biochemical procedures were performed as described (Boucard et al., 2005; Chubykin et al., 2007). Recombinant proteins were produced in transfected HEK293T cells (Ushkaryov et al., 1994). Constructs were generated as described in detail in the Supplemental Data. Antibodies and other reagents were purchased commercially or described previously (Boucard et al., 2005; Chubykin et al., 2007; for a detailed description, see Supplemental Data).

Statistics

All data are expressed as means ± SEMs; all experiments were performed with at least three independent cultures, and evaluated statistically by Student's t test (n = culture numbers). Detailed methods for all procedures are described in the Supplemental Data, and all numerical data are listed in Table S1 available online.

SUPPLEMENTAL DATA

Supplemental data for this article include four figures, one table, and Supplemental Experimental Procedures and can be found at http://www.cell.com/neuron/supplemental/S0896-6273(09)01002-2.

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