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Leucine-Rich Repeat Transmembrane Proteins Are Essential for Maintenance of Long-term Potentiation

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SUMMARY

Leucine-rich repeat transmembrane proteins (LRRTMs) are synaptic cell adhesion molecules that trigger excitatory synapse assembly in cultured neurons and influence synaptic function *in vivo*, but their role in synaptic plasticity is unknown. shRNA-mediated knockdown (KD) of LRRTM1 and -2 *in vivo* in CA1 pyramidal neurons of newborn mice blocked long-term potentiation (LTP) in acute hippocampal slices. Molecular replacement experiments revealed that the LRRTM2 extracellular domain is sufficient for LTP, likely because it mediates binding to neurexins (Nrxs). Examination of surface expression of endogenous AMPA receptors (AMPARs) in cultured neurons suggests that LRRTMs maintain newly delivered AMPARs at synapses following LTP induction. LRRTMs are also required for LTP of mature synapses on adult CA1 pyramidal neurons indicating that the block of LTP in neonatal synapses by LRRTM1 and -2 KD is not due to impairment of synapse maturation.

INTRODUCTION

Neurons use complex mechanisms that allow activity patterns to regulate the complement of AMPARs at synapses. LTP at excitatory synapses on hippocampal CA1 pyramidal cells remains the most compelling and extensively studied model of such synaptic plasticity (Bliss and Collingridge, 1993; Malenka and Bear, 2004). Despite decades of mechanistic work on this phenomenon and the general consensus that it involves an increase in the

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SUPPLEMENTAL INFORMATION
Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found online.

AUTHOR CONTRIBUTIONS
G.J.S-L. constructed plasmids, generated lentiviruses, injected these in P0 mice and performed and analyzed agonist-evoked currents in outside out patches. G.J.S-L. and W.M. performed and analyzed long-term plasticity experiments in acute slices and injected lentiviruses in P21 mice. P.A. performed and analyzed all GluA1 surface expression assays in hippocampal cultures. M.A performed immunoprecipitation assay. G.J.S-L., P.A., T.C.S. and R.C.M. wrote the manuscript and all authors approved the final version.

number of synaptic AMPARs (Bredt and Nicoll, 2003; Collingridge et al., 2004; Malinow and Malenka, 2002; Shepherd and Huganir, 2007), the mechanisms underlying the trafficking of AMPARs to the synapse and their stabilization within the postsynaptic density (PSD) during LTP remain controversial and poorly understood.

LTP may involve several mechanistically distinct steps: exocytosis of AMPARs into the plasma membrane at peri- or extra-synaptic sites, lateral diffusion of perisynaptic AMPARs into the PSD and direct or indirect trapping of these AMPARs within the PSD (Henley et al., 2011; Kennedy and Ehlers, 2006; Opazo and Choquet, 2010; Opazo et al., 2012). Although manipulations of membrane-associated guanylate kinases (MAGUKs) such as PSD95, which are prevalent proteins in the PSD, have effects on basal excitatory synaptic transmission (Elias and Nicoll, 2007), their necessity in mediating the increase in synaptic strength during LTP is unclear. Functional redundancy among the MAGUKS may explain the finding that removal or reduction of individual MAGUKs does not significantly impair LTP (Carlisle et al., 2008; Cuthbert et al., 2007; Ehrlich et al., 2007; Howard et al., 2009; Migaud et al., 1998). However, other hypotheses are equally plausible. Notably, recent findings suggest that the mechanisms controlling the delivery and maintenance of synaptic AMPARs in basal conditions and during LTP may be distinct (Adesnik et al., 2005; Ahmad et al., 2012; Jurado et al., 2013; Sumioka et al., 2011).

Synaptic cell adhesion proteins are involved in the formation, maturation and specification of synapses (Dalva et al., 2007; Missler et al., 2012; Siddiqui and Craig, 2011). Neuroligins (NLs) have attracted particular attention because of their synaptogenic actions when overexpressed and their genetic association with neuropsychiatric disorders (Craig and Kang, 2007; Krueger et al., 2012; Südhof, 2008). Although KD or knockout (KO) of NL1 can impair LTP, this effect may be due to the associated reduction of NMDA receptor-mediated currents and spine calcium influx (Blundell et al., 2010; Chubykin et al., 2007; Kim et al., 2008; Kwon et al., 2012). Recently, KD of NL1 has been reported to impair LTP in dentate gyrus granule cells and neonatal CA1 pyramidal cells independent of an effect on NMDARs, but not at synapses on mature CA1 pyramidal cells, possibly because the LTP deficit due to NL1 KD occurs only at recently formed, immature synapses (Shipman and Nicoll, 2012).

Like NLs, LRRTMs are synaptogenic *in vitro*, potently bind to presynaptic Nrxs and are associated with neuropsychiatric disorders (de Wit et al., 2009; Francks et al., 2007; Ko et al., 2009; Linhoff et al., 2009; Siddiqui et al., 2010; Sousa et al., 2010). However, the functional role of LRRTMs at synapses is just beginning to be explored. LRRTMs comprise a family of four (LRRTM1-4) homologous, type I transmembrane proteins with differential distribution within the brain (Laurén et al., 2003). While the KD of LRRTM1 and/or -2 *in vitro* does not cause a change in synapse numbers (Ko et al., 2011), and LRRTM KDs *in vitro* and *in vivo* have yielded somewhat inconsistent results, decreases in AMPAR surface expression *in vitro* and AMPAR-mediated synaptic transmission *in vivo* have been observed (de Wit et al., 2009; Soler-Llavina et al., 2011). Furthermore, LRRTMs may directly bind to AMPAR subunits both *in vitro* and *in vivo* (de Wit et al., 2009; Schwenk et al., 2012).

Here we used *in vivo*, viral-mediated KD of LRRTM1 and -2 (double knockdown, DKD) to examine the role of LRRTMs in LTP at excitatory synapses on CA1 pyramidal neurons in mouse hippocampus. LRRTM DKD blocked or dramatically impaired LTP in neonatal (P14–18) and mature (P35–39) CA1 pyramidal neurons, respectively. Molecular replacements with recombinant LRRTM2 revealed that its extracellular LRR domain, likely via interactions with Nrxs, is critical for LRRTM function in LTP. Assays of AMPAR surface expression in cultured hippocampal neurons suggest that LRRTMs are required for the stabilization of AMPARs at synapses following LTP induction. These results reveal an

unexpected role for LRRTMs in LTP at both young and mature synapses and are consistent with a model in which LRRTMs are required for maintaining or trapping AMPARs at synapses during the initial phase of LTP.

RESULTS

In Vivo DKD of LRRTM1 and -2 Blocks LTP

To explore the role of LRRTMs in NMDAR-dependent LTP, we used well-characterized shRNAs that in dissociated cultured neurons suppress endogenous mRNAs for LRRTM1 and -2, the two isoforms highly expressed in CA1 (Laurén et al., 2003), by ~90% and ~75% respectively (Ko et al., 2011; Soler-Llavina et al., 2011). A lentivirus expressing the shRNAs and GFP was injected into the hippocampus of postnatal day 0 (P0) wild type mice (Figure 1A). Acute slices were prepared 14–18 days post-infection and whole cell recordings from CA1 pyramidal neurons were made (Figures 1B and 1C). While control neurons in slices prepared from infected animals exhibited robust LTP (Figures 1D and 1E; 1.62 ± 0.23 of baseline 46–50 min. after induction, n = 8), LTP was blocked in DKD neurons expressing the LRRTM1 and -2 shRNAs (Figures 1D and 1E; 0.99 ± 0.1, n = 19). Similar to other manipulations that block LTP (Malenka and Nicoll, 1993), DKD neurons exhibited an initial potentiation that returned to baseline over 40–50 minutes. To determine whether LRRTM1 and -2 have a specific role in LTP, we assessed the effect of the DKD on NMDAR-dependent long-term depression (LTD). LTD in DKD and uninfected control neurons was identical (Figures 1F and 1G; Control = 0.49 ± 0.06, n = 9; DKD = 0.48 ± 0.04, n = 10), a result that is consistent with the lack of an effect of the DKD on NMDAR-mediated synaptic responses (Soler-Llavina et al., 2011). These results suggest that LRRTMs have a critical, requisite role in LTP and that the block of LTP by DKD is not due to an impairment in the induction of LTP.

To test whether the block of LTP by LRRTM DKD might be due to off-target effects of the shRNAs, we performed experiments in which we replaced LRRTM1 and -2 with an shRNA-resistant version of LRRTM2 (DKD-LRR2) (Ko et al., 2011; Soler-Llavina et al., 2011). [We did not attempt rescue experiments with LRRTM1 because overexpressed recombinant LRRTM1 accumulates in the endoplasmic reticulum and traffics poorly to the plasma membrane (Francks et al., 2007; Linhoff et al., 2009).] LTP was rescued by expression of shRNA-resistant LRRTM2 along with the shRNAs (Figures 2A and 2B; Control = 1.57 ± 0.19, n = 10; DKD-LRR2 = 1.55 ± 0.15, n = 14). To interpret such rescue experiments, it is important to determine whether overexpression of the protein of interest alone has any measurable phenotype. Thus, we overexpressed LRRTM2 (LRR2OE; Figure 2C) and found no effect on LTP when compared to interleaved control neurons (Figure 2D; Control = 1.79 ± 0.20, n = 9; LRR2OE = 1.79 ± 0.18, n = 10). These results indicate that the block of LTP caused by the LRRTM DKD is specific to the loss of LRRTM1 and -2.

The Extracellular Domain of LRRTMs is Required for LTP

When LRRTMs are overexpressed, their extracellular domains are necessary and sufficient for their ability to promote synaptogenesis both in non-neuronal cells and cultured neurons (de Wit et al., 2009; Linhoff et al., 2009). Moreover, LRRTM2, via its extracellular domain co-immunoprecipitates with the AMPAR subunits GluA1 and GluA2 in an *in vitro*, overexpression system (de Wit et al., 2009). To determine the domain of LRRTMs that is important for LTP, we expressed the extracellular domain of LRRTM2 (fused to the transmembrane domain of the platelet-derived growth factor receptor; Figure 2E; DKD-LRR2Ex) (Ko et al., 2011; Soler-Llavina et al., 2011). Replacement of endogenous LRRTMs with LRR2Ex resulted in LTP that was comparable to the LTP measured in

interleaved control cells from the same sets of slices (Figure 2F; Control = 1.57 ± 0.19, n = 10 cells; DKD-LRR2Ex = 1.39 ± 0.14, n = 15 cells).

The extracellular domain of LRRTMs binds Nrxs with high affinity (de Wit et al., 2009; Ko et al., 2009; Siddiqui et al., 2010), an interaction that may be necessary for axons to make synaptic contacts onto non-neuronal cells expressing LRRTM2 (Ko et al., 2011; Siddiqui et al., 2010). To test whether LRRTM function in LTP requires binding to Nrxs, we introduced two mutations (D260A, T262A) reported to prevent LRRTM-Nrx interaction (Siddiqui et al., 2010), into the LRR2Ex replacement construct (Figure 2G; DKD-LRR2Ex^{AA}). Cells expressing DKD-LRR2Ex^{AA} exhibited dramatically reduced LTP relative to interleaved controls (Figure 2H; Control = 1.77 ± 0.16, n = 14 cells; DKD-LRR2Ex^{AA} = 1.12 ± 0.14, n = 21 cells). Importantly, the overexpressed LRR2Ex^{AA} reached the neuronal cell surface and co-localized with vGluT1 (Figures S1 and S2) suggesting that the mutations in LRR2Ex^{AA} do not completely block LRRTM2 delivery to the plasma membrane and its synaptic localization. The lack of LTP rescue by LRR2Ex^{AA} could also be due to disruption of the binding of LRRTM2 to AMPARs. To test this possibility, we co-expressed FLAG-tagged GluA1 with mVenus-tagged, full length LRRTM2 or LRRTM2^{AA} in HEK 293T cells and examined their interaction by immunoprecipitation (Figure S3). GluA1-FLAG co-immunoprecipitated equally well with both wild type LRRTM2 and mutant LRRTM2^{AA} suggesting that the mutations do not disrupt the association between LRRTM2 and GluA1. These results demonstrate a critical role for the extracellular LRR domain of LRRTMs, likely due to its interaction with Nrxs, in LTP.

LRRTMs Stabilize Synaptic AMPARs in Cultured Neurons

Changes in synaptic responses in slices do not necessarily directly reflect changes in endogenous surface AMPARs. Therefore, to test the role of LRRTM1 and -2 in NMDAR-triggered delivery of endogenous AMPARs to the cell surface, we used a neuronal culture model of LTP in which activation of NMDARs leads to an increase in surface AMPARs at synapses (termed cLTP for chemical LTP) (Ahmad et al., 2012; Jurado et al., 2013; Lu et al., 2001; Park et al., 2004; Passafaro et al., 2001). We infected cultured hippocampal neurons at 8 days-*in-vitro* (DIV8) with control (GFP alone), DKD or DKD-LRR2 lentiviruses. At DIV 16–18, we briefly (3 min) incubated these neurons with a control or cLTP solution. After 20 min, neurons were fixed, immunostained for surface AMPARs containing GluA1 and imaged with confocal microscopy (Figure 3A) (Ahmad et al., 2012; Jurado et al., 2013 and Supplemental Experimental Procedures). In control cells the cLTP solution caused a clear increase in total surface expression of AMPARs (Figures 3A and 3B; control = 100 ± 7.0%, n = 41; control + cLTP = 194.5 ± 13.1%, n = 39). LRRTM DKD in cultured neurons produced two major effects: an increase in basal surface levels of AMPARs and a significant reduction in surface AMPARs following cLTP (Figures 3A and 3B; DKD = 169.6 ± 25.3%, n = 45; DKD + cLTP = 110.9 ± 16.5%, n = 45). Both phenotypes were reversed by the simultaneous expression of LRRTM2 (Figures 3A and 3B; DKD-LRR2 = 102.1 ± 7.8%, n = 48; DKD-LRR2 + cLTP = 184.6 ± 9.8%, n = 48). The increase in surface GluA1 caused by LRRTM DKD in basal conditions is unlikely due to an upregulation of GluA1 expression since the total pool of GluA1-containing AMPARs (surface + internal) was unaffected (Figure S4).

The finding that LRRTM DKD increased basal levels of surface AMPARs is difficult to reconcile with previous results reporting that this same DKD *in vivo* in neonatal animals selectively reduced AMPAR-mediated synaptic currents (Soler-Llavina et al., 2011). Furthermore, LRRTM2 KD alone was reported to decrease GluA1 puncta density in cultured hippocampal neurons (de Wit et al., 2009) although the specificity of the shRNA used in this study has been questioned (Ko et al., 2011). A hypothesis that can reconcile these results and also account for the block of LTP by LRRTM DKD is that LRRTMs

contribute to the stabilization of AMPARs at synapses and their absence results in an accumulation of extrasynaptic AMPARs perhaps at the expense of synaptic ones. To test these hypotheses we quantified the relative levels of synaptic surface AMPARs, defined as GluA1 puncta that colocalized with the vesicular glutamate transporter vGluT1. Under basal conditions, LRRTM DKD caused a decrease in the proportion of GluA1 puncta found at synapses (Figure 3D; Control = 83.6 ± 2.14%, n = 20; DKD = 55.12 ± 3.85, n = 21) as well as a decrease in the average intensity of GluA1 staining at synaptic puncta (Figure 3E; Control = 9.5 ± 1.16, n = 20; DKD = 6.0 ± 0.68, n = 21). Consistent with the increase in total surface GluA1 caused by LRRTM DKD (Figure 3B), this manipulation caused an increase in average puncta intensity when both synaptic and extrasynaptic puncta were included (Figure 3F; Control = 7.6 ± 1.62, n = 20; DKD = 16.9 ± 2.10, n = 21). Inducing cLTP increased synaptic and total GluA1 puncta intensity in control cells while causing a decrease in total, but not synaptic GluA1 intensity in LRRTM DKD cells (Figures 3E and 3F; Control: Synaptic = 16.2 ± 1.42; Total = 16.4 ± 1.80; DKD: = 16.9 ± 2.10, n = 22; DKD: Synaptic = 6.8 ± 0.63; Total = 9.4 ± 1.5; n = 21). All of these measurements returned to control values when shRNA-resistant LRRTM2 was also expressed (Figures 3C-3F; % Synaptic GluA1: Basal = 86.0 ± 1.27, +cLTP = 88.8 ± 1.58. Synaptic intensity = 9.7 ± 1.34, +cLTP = 18.9 ± 1.74. Total intensity = 7.5 ± 0.99, +cLTP = 15.4 ± 1.33; n = 24). These results are consistent with the hypothesis that LRRTMs are required to maintain a population of AMPARs at synapses and that their reduction results in a concomitant decrease of synaptic and increase in extrasynaptic AMPARs.

To further test whether LRRTM DKD causes an increase in the levels of extrasynaptic surface AMPARs, we measured AMPAR-mediated currents evoked by fast glutamate application in somatic, outside out patches (Figure 3G) obtained from cultured neurons expressing either GFP alone or the LRRTM shRNAs. The current amplitude measured in patches from LRRTM DKD neurons was significantly larger than in control patches (Figure 3H; Control = 197.8 ± 23.9, n = 23; DKD = 301 ± 36.4, n = 25). These data provide an independent measure supporting the conclusion that LRRTM1 and -2 DKD results in an increase in the levels of extrasynaptic surface AMPARs.

The hypothesis that LRRTMs are required for maintaining recently delivered AMPARs at synapses during LTP predicts that initial delivery of AMPARs to the plasma membrane shortly after LTP induction should not be impaired. To test this prediction, we examined surface GluA1 at two different time points following cLTP induction in control, DKD and DKD-LRR2 cultured neurons (Figures 3I, 3J and S5). At 10 minutes there was a comparable increase in surface GluA1 expression in all experimental groups despite the fact that the LRRTM DKD again caused an increase in basal surface levels of GluA1 (Figure 3I, 3J and S5; Control, 100 ± 16.2%, n = 21; Control + cLTP, 191.3 ± 21.2%, n = 26; DKD, 150.0 ± 14.5%, n=26; DKD + cLTP, 214.2 ± 27.8, n = 20; DKD-LRR2, 101 ± 12.0%, n = 25; DKD-LRR2 + cLTP, 164.8 ± 28.0%, n = 25). Importantly, at this 10 min time point in all groups, a clear increase in surface GluA1 level at synapses was detected (Figure S6). Finally, consistent with our previous experiments (Figures 3A–3D), in these same sets of cultures 20 minutes post cLTP induction, surface GluA1 expression was decreased by the LRRTM DKD whereas it was increased in both control and DKD-LRR2 neurons (Figures 3I and 3J; Control, 100 ± 19.7%; Control + cLTP, 239.2 ± 32.7%; DKD, 168.7 ± 16.1%; DKD + cLTP, 114.2 ± 22.3%; DKD-LRR2, 98.5 ± 17.6%, DKD-LRR2 + cLTP, 166.3 ± 26.7; n = 20–26 for each condition).

These results demonstrate that LRRTM DKD does not impair the initial delivery of AMPARs to the plasma membrane and synapses soon after cLTP induction and that the block of LTP at later time points is due to the lack of retention of newly delivered AMPARs to synapses. The decrease in total, but not synaptic, surface GluA1 following glycine

treatment in the DKD cells suggests that the extrasynaptic AMPARs in the DKD cells may be relatively unstable and more susceptible to endocytosis. Consistent with this hypothesis, the constitutive endocytosis of GluA1-containing AMPARs increased following LRRTM DKD and returned to basal levels with expression of LRRTM2 (Figure S7).

LRRTMs Are Required for LTP at Mature Synapses

Our results suggest that LRRTMs are required for LTP at synapses on early postnatal CA1 pyramidal neurons *in vivo* and on cultured neurons *in vitro*. However, the effects of LRRTM DKD on basal AMPAR-mediated synaptic responses *in vivo* depends on the maturational state of the synapses (Soler-Llavina et al., 2011). Furthermore, NL1 KD was reported to impair LTP at early postnatal but not at mature synapses on CA1 pyramidal neurons (Shipman and Nicoll, 2012), although the NL1 KO does not cause a major impairment in LTP (Blundell et al., 2010), suggesting that NL1 is not required during development to render synapses competent for LTP. These findings raise the possibility that LRRTMs may not play a critical role in mediating LTP at mature synapses but instead that the *in vivo* LRRTM DKD at P0 may prevent synapses from reaching a maturational state necessary to support LTP. To address this possibility, we injected the LRRTM DKD lentivirus into the CA1 region of P21 mice, a time point at which synapses have largely matured, and then performed recordings in slices prepared 14–18 days later (Figures 4A and 4B). P35–39 control neurons expressed robust LTP (Figure 4C) whereas LTP was dramatically reduced in DKD neurons (Figures 4D and 4E; Control = 2.1 ± 0.18, n = 13 cells; DKD = 1.26 ± 0.11, n = 12 cells). Furthermore, expression of LRRTM2 rescued LTP (Figures 4F and 4G; DKD-LRR2 = 2.0 ± 0.30, n = 7 cells) as did expression of LRR2Ex (Figures 4H and 4I; Control = 2.14 ± 0.41, n = 5 cells; DKD-LRR2Ex = 2.08 ± 0.33, n = 6 cells).

DISCUSSION

Despite decades of effort, the molecular mechanisms underlying classic NMDAR-dependent LTP at excitatory synapses on hippocampal CA1 pyramidal neurons remain poorly understood. Indeed, recent work points out the need to re-examine current hypotheses about LTP mechanisms (Granger et al., 2012; Lee et al., 2013; Volk et al., 2013) and the importance of testing the role of novel proteins. Here we investigated the role of LRRTMs (Laurén et al., 2003; Linhoff et al., 2009) in standard LTP because, like NLs, LRRTMs form an adhesion complex with Nrxs (de Wit et al., 2009; Ko et al., 2009, 2011; Siddiqui et al., 2010), their *in vivo* KD during early postnatal development affects AMPAR-, but not NMDAR-mediated synaptic responses (Soler-Llavina et al., 2011), and they may directly bind to AMPAR subunits (de Wit et al., 2009; Schwenk et al., 2012). We find that LRRTM1 and -2 DKD *in vivo* blocks LTP in neonatal CA1 pyramidal neurons, a deficit that is rescued by wildtype LRRTM2. Further replacement experiments revealed that the extracellular, but not intracellular, domain of LRRTM2 is required for LTP. LTP was not rescued by expression of a mutant LRRTM2 reported to impair binding to Nrxs (Siddiqui et al., 2010), although whether this mutant quantitatively reaches the surface to the same degree as wildtype LRRTM2 is unknown. Importantly, LRRTM1 and-2 DKD in adult CA1 pyramidal neurons *in vivo* also strongly impaired LTP. These results demonstrate that the block of LTP by LRRTM1 and -2 DKD is not due to some unknown effect on synapse maturation, but rather due to a critical role of LRRTMs in LTP at mature synapses.

A cell culture model of LTP provided further insight into the mechanisms by which LRRTMs may function in LTP. LRRTM1 and-2 DKD blocked this model of LTP and surprisingly increased the net surface expression of AMPARs under basal conditions. Immunocytochemical and electrophysiological assays revealed that DKD caused an increase in surface expression of extrasynaptic AMPARs while decreasing synaptic AMPARs. Furthermore, the DKD did not affect the initial increase in surface and synaptic AMPAR

expression 10 minutes after cLTP induction yet caused a decrease in net AMPAR surface expression when measured 20 minutes following cLTP. All of the effects of the DKD in cultured neurons were reversed by wildtype LRRTM2 suggesting that the phenotypes were not due to off-target effects.

The results in cultured neurons are consistent with the decrease in AMPAR-mediated synaptic transmission caused by LRRTM DKD *in vivo* in neonatal hippocampus (de Wit et al., 2009; Soler-Llavina et al., 2011) as well as the time course of the block of LTP in acute slices. They support the hypothesis that LRRTMs are required for maintaining a normal complement of synaptic AMPARs to support basal synaptic transmission but not for the AMPAR exocytosis that occurs following LTP induction. However, in adult CA1 pyramidal neurons, LRRTM1 and -2 DKD did not have a detectable effect on basal AMPAR-mediated synaptic transmission (Soler-Llavina et al., 2011). A simple hypothesis to explain all of these results is that in young, developing synapses LRRTMs serve two functions. They help maintain a normal complement of synaptic AMPARs for basal synaptic transmission and following LTP induction, they contribute to the scaffolding or "slot" complex that stabilizes the newly delivered AMPARs (Malinow and Malenka, 2002; Opazo and Choquet, 2010). In their absence following LTP induction, AMPARs transiently diffuse into but cannot be maintained within the PSD; they escape to sites at which endocytosis occurs, a process that may have been accelerated by the LTP induction protocol. Indeed, while the LTP induction protocol in slices rarely elicited LTD in control cells, it often elicited LTD in cells in which LRRTM1 and-2 were knocked down or replaced with the mutant LRR2Ex^{AA} (see cumulative fraction graphs in Figures 1E and 2H). The lack of effect of LRRTM DKD on basal synaptic transmission in adult CA1 pyramidal neurons (Soler-Llavina et al., 2011) suggests that at mature synapses, LRRTMs either do not play a role in maintaining a complement of AMPARs to support basal synaptic transmission or that other molecules can compensate for their loss. Nonetheless, our results support the hypothesis that LRRTMs are required for stabilizing newly delivered AMPARs during at least the first 40–50 min of LTP in both developing and mature synapses.

The detailed molecular interactions by which LRRTMs may stabilize AMPARs at synapses during LTP are unknown. LRRTMs can directly interact with AMPAR subunits (de Wit et al., 2009; Schwenk et al., 2012), and recent work supports the hypothesis that binding of LRRTMs to presynaptic Nrxs is critical for their maintenance and perhaps function, at synapses (Aoto et al., 2013). Specifically, constitutive genetic inclusion of splice site 4 in Nrx3, which prevents Nrx binding to LRRTMs (Ko et al., 2009), resulted in decreases in basal AMPAR synaptic content, a block of LTP, an enhancement of constitutive AMPAR endocytosis, and a ~45% decrease in surface levels of LRRTM2 (Aoto et al., 2013). Thus, the synaptic deficits caused by inclusion of splice site 4 in Nrx3 are remarkably similar to those caused by LRRTM DKD, suggesting that a critical trans-synaptic protein complex required for maintaining AMPARs at synapses may involve presynaptic Nrx interactions with postsynaptic LRRTMs.

EXPERIMENTAL PROCEDURES

Detailed experimental procedures can be found in Supplemental Experimental Procedures online.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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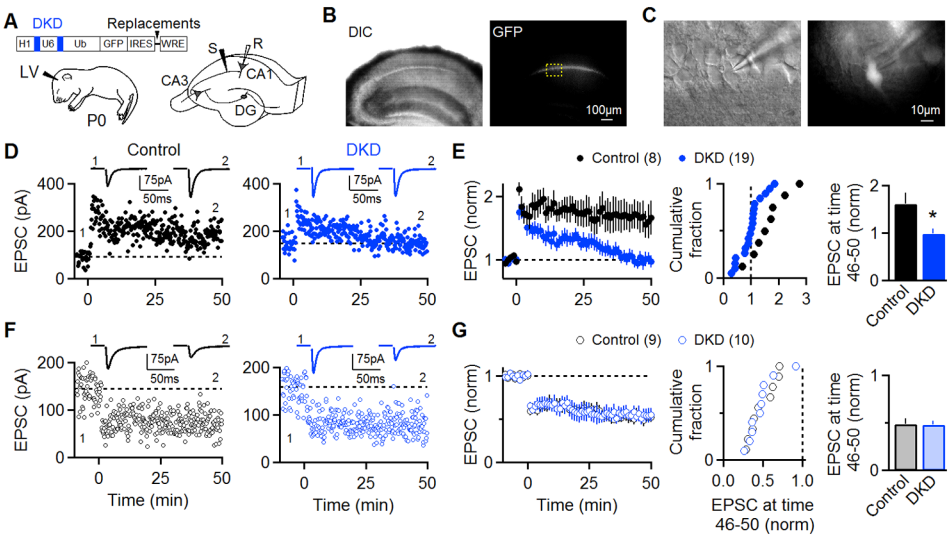

Figure 1.

In vivo LRRTM1 and -2 Double Knockdown (DKD) in Neonatal CA1 Pyramidal Neurons Blocks LTP. (A) Schematic of shRNA and replacement lentiviral backbone and the experimental approach for LTP experiments in young mice. (B) Low magnification images of an acute hippocampal slice in DIC (left) and epifluorescence (right) modes showing specific CA1 infection. (C) High magnification (60x) images of the boxed region in (B) showing mosaic GFP expression and a patched CA1 pyramidal neuron. (D) Time courses of representative LTP experiments for control (left) and DKD neurons (right). Averages of 30 consecutive EPSCs during the baseline (1) and 46–50 minutes after tetanic stimulation (2, delivered at time 0) are shown above each graph. (E) Summary time course (left), cumulative fraction of all experiments in the set (middle) and quantification of the LTP magnitude (right) for DKD cells and interleaved controls. In this and all subsequent figures, summary data is presented as mean ± SEM and numbers in parentheses represent number of cells. * $p < 0.01$. (F–G) As in (D–E), for LTD experiments.

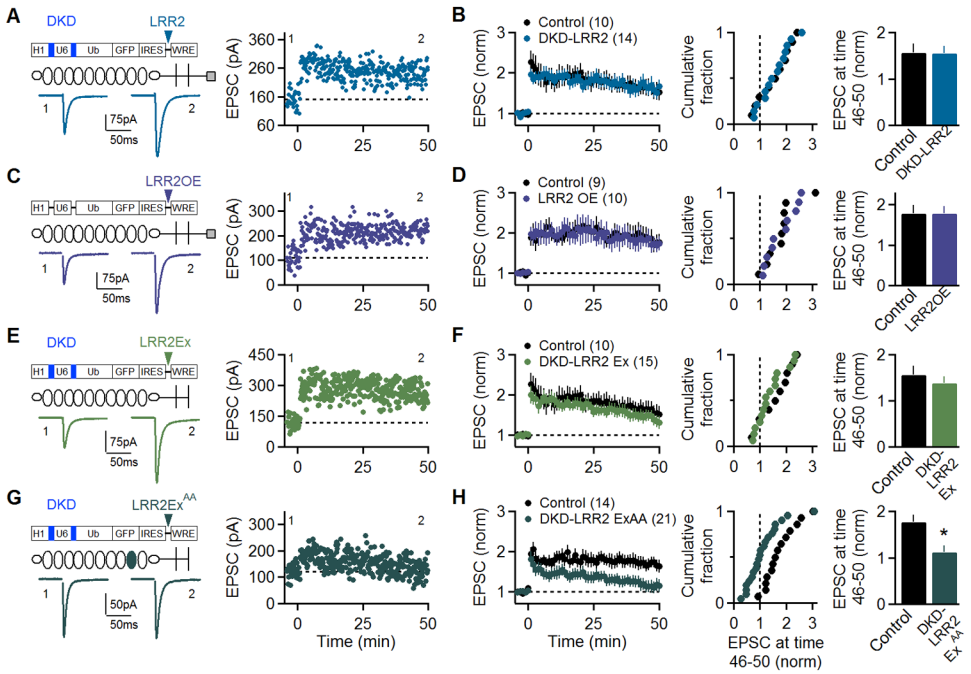

Figure 2.

The Extracellular Domain of LRRTM2 is Sufficient for its Function in LTP. (A, C, E, G) Diagrams of the lentiviral vector and recombinant LRRTM2 constructs used for molecular replacement and over expression experiments (top left). Sample, average EPSCs during baseline and after LTP expression (bottom left) and time course (right) of representative, single LTP experiments following the indicated molecular manipulations. (B, D, F, H) Summary time course (left), cumulative fraction of all experiments in the set (middle) and quantification of the LTP magnitude (right) for molecularly-manipulated and corresponding interleaved control neurons. The DKD-LRR2 (A) and DKD-LRR2Ex (E) sets were performed in parallel and therefore share the same control group. For clarity, and to facilitate visual comparison, this control group was plotted in both panels (B and F). *p < 0.01 (See also Figure S1–S3.)

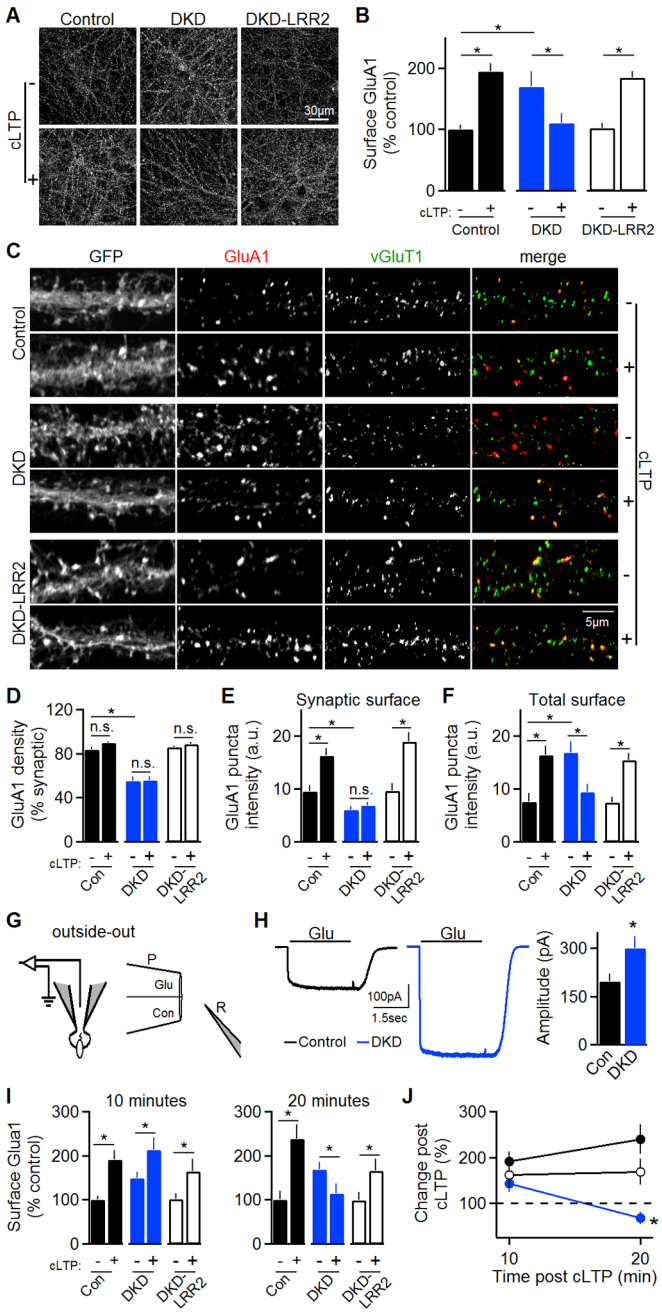

Figure 3.

LRRTM1 and -2 DKD Prevents cLTP and Alters Surface GluA1 Expression in Cultured Neurons.(A) Representative images from hippocampal neuronal cultures infected with lentiviruses expressing the indicated constructs and immunostained for the AMPAR subunit GluA1 20 minutes after treatment with control (−cLTP) or glycine-containing solution (+cLTP). (B) Summary graph showing surface GluA1 levels in the three sets of cultures in basal conditions (−cLTP) and following cLTP (+cLTP). Bars represent mean ± SEM. $p < 0.0001$. (C) Representative images of dendrites from cultured neurons infected with lentiviruses expressing the indicated constructs and immunostained for GFP, GluA1 and vGluT1 in basal conditions and following cLTP. (D–F) Summary quantification of the

percentage of GluA1 puncta that are synaptic (D) and the intensity of synaptic (E) and total (F) GluA1 puncta in basal conditions and following cLTP. (*$p < 0.001$). (G) Schematic of the outside-out voltage clamp configuration and fast glutamate perfusion set up. P = perfusion pipette, R = recording pipette, Con = control solution (ACSF), Glu = ACSF + glutamate, AP-5 and cyclothiazide. (H) Representative glutamate-evoked currents obtained from control and DKD patches (left) and summary quantification (right) of currents. (I) Quantification of surface GluA1 levels in neurons expressing GFP, DKD and DKD-LRR2 in basal conditions and following cLTP induction. An increase in surface GluA1 levels can be detected in DKD neurons 10 but not 20 minutes after cLTP induction. (J) Quantification of the change in relative GluA1 surface levels following cLTP at both time points. (*$p < 0.05$) (See also Figures S4–S7.)

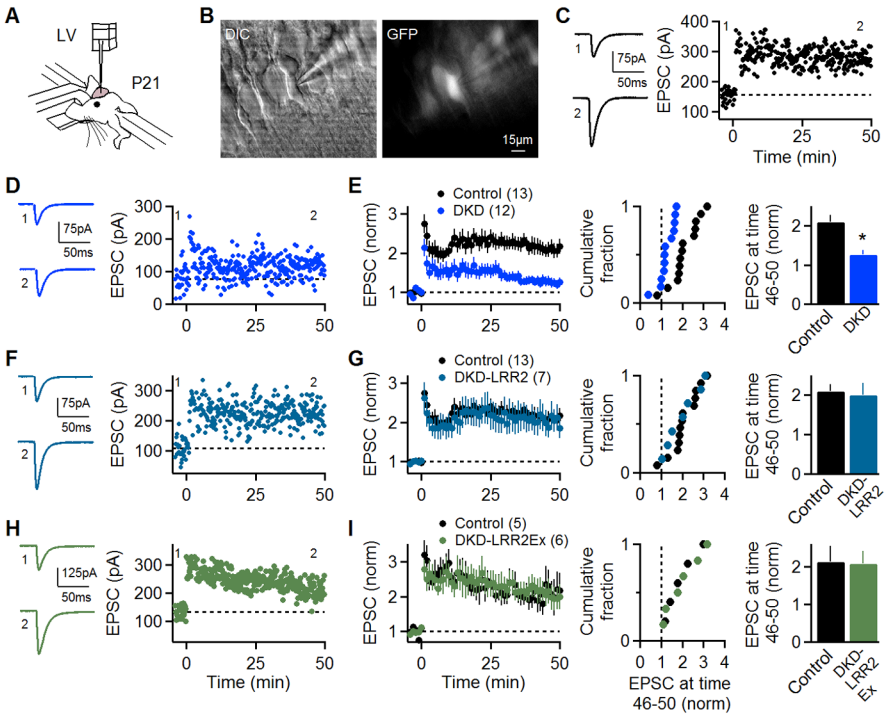

Figure 4. *In Vivo* LRRTM DKD Impairs LTP in Young Adult Hippocampus

(A) Diagram showing a mouse on stereotaxic apparatus for injection of lentiviruses at P21. (B) High magnification (60x) of CA1 pyramidal neurons in acute hippocampal slice in DIC (left) and epifluorescence (right) modes showing a patch pipette on an infected neuron from which a whole recording was obtained. (C, D, F, H) Representative EPSCs (left) and time courses (right) of LTP experiments obtained from a control neuron (C) and neurons infected with DKD (D), DKD-LRR2 (F) and DKD-LRR2Ex (H) lentiviruses, respectively. (E, G, I) Summary time course (left), cumulative fraction of all experiments in the set (middle) and quantification of the LTP magnitude (right) for neurons expressing the indicated constructs and the corresponding controls. The DKD and DKD-LRR2 manipulations (D and F) were performed in parallel and share the same group of control neurons. For clarity and to facilitate visual comparison, these control data are plotted in both panels E and G. *p < 0.001