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Amyloid- β Oligomers Interact with Neurexin and Diminish Neurexin-mediated Excitatory Presynaptic Organization

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Alzheimer's disease (AD) is characterized by excessive production and deposition of amyloid-beta ($A\beta$) proteins as well as synapse dysfunction and loss. While soluble $A\beta$ oligomers ($A\beta$ Os) have deleterious effects on synapse function and reduce synapse number, the underlying molecular mechanisms are not well understood. Here we screened synaptic organizer proteins for cell-surface interaction with $A\beta$ Os and identified a novel interaction between neurexins (NRXs) and $A\beta$ Os. $A\beta$ Os bind to NRXs via the N-terminal histidine-rich domain (HRD) of β -NRX1/2/3 and alternatively-spliced inserts at splicing site 4 of NRX1/2. In artificial synapse-formation assays, $A\beta$ Os diminish excitatory presynaptic differentiation induced by NRX-interacting proteins including neuroligin1/2 (NLG1/2) and the leucine-rich repeat transmembrane protein LRRTM2. Although $A\beta$ Os do not interfere with the binding of NRX1 β to NLG1 or LRRTM2, time-lapse imaging revealed that $A\beta$ O treatment reduces surface expression of NRX1 β on axons and that this reduction depends on the NRX1 β HRD. In transgenic mice expressing mutated human amyloid precursor protein, synaptic expression of β -NRXs, but not α -NRXs, decreases. Thus our data indicate that $A\beta$ Os interact with NRXs and that this interaction inhibits NRX-mediated presynaptic differentiation by reducing surface expression of axonal β -NRXs, providing molecular and mechanistic insights into how $A\beta$ Os lead to synaptic pathology in AD.

Alzheimer's disease (AD) is characterized by the accumulation of toxic amyloid- β ($A\beta$)-peptides, the principal constituent of plaques in the brains of AD patients^{1,2}. In addition, loss of synapses in the brain is an early pathological feature of AD and is the best correlate of cognitive impairment^{3,4}. Experimentally, $A\beta$ oligomers ($A\beta$ Os) cause synapse loss and synapse dysfunction both *in vitro* and in mouse models of AD⁴⁻⁶. The *in vitro* experiments show that the application of soluble $A\beta$ Os to hippocampal slices or cultured neurons decreases immunoreactivity for pre- and post-synaptic proteins⁷⁻¹² and the density of dendritic spines^{9,13-16}. Further, $A\beta$ treatment of hippocampal slices distorts synaptic plasticity^{4-6,17}. Specifically, $A\beta$ treatment blocks long-term potentiation (LTP) and enhances long-term depression (LTD). Thus, synapses exhibit vulnerability to $A\beta$. However, little is known about the molecular mechanisms underlying this vulnerability.

Synaptic organizing complexes are trans-synaptic adhesion molecules with an ability to promote pre- and/or post-synaptic organization (hereinafter synaptogenic activity) and are thought to function as essential molecular signals for synapse formation, maturation, maintenance, and plasticity¹⁸⁻²². The neuroligin (NLG)-neurexin (NRX) complex is one of the most well-studied synaptic organizing complexes, and mutations in this complex are genetic determinants predisposing to cognitive disorders such as autism and schizophrenia^{18,19,21}. Recent studies have identified several other synaptic organizing complexes including TrkC-PTP σ ²³, Slitrk-PTP δ ^{24,25}, the leucine-rich repeat transmembrane protein (LRRTM) 1/2/3-NRX²⁶⁻³⁰, calyculin3- α -NRX³¹, GluR δ -cerebellin-NRX³²⁻³⁴, NGL3-LAR^{35,36}, and IL1RAPL1/IL1RacP-PTP δ ³⁷⁻³⁹. Thus there are many synaptic organizing proteins, any of which could be targets for $A\beta$ in synapse disruption.

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Indeed, there is recent evidence that A β pathogenic processes may affect synaptic organizers. Several organizers including NRXs^{40,41}, leukocyte antigen-related tyrosine phosphatase (LAR)^{42,43} and NLG1⁴⁴ are cleaved by proteinases involved in the generation of A β , indicating that their function may be altered coordinately with A β production. Also, A β Os bind to soluble NLG1 ectodomain⁴⁵. These results emphasize the importance of understanding whether and how A β Os affect the physiological roles of synaptic organizing complexes. But, there has been no published study that has systematically tested for physical and functional interactions of synaptic organizing complex proteins with A β Os.

In this study, we performed cell surface binding assays using soluble A β Os to screen for their interaction with synaptic organizers expressed at the cell surface. We found that A β Os interact with NRX family members and defined their A β O-binding domains. A β Os diminish NRX-mediated presynaptic organization by decreasing β -NRX expression on the axonal surface, although this does not affect NRX-NLG1 interaction or NRX-LRRTM2 interaction. In a transgenic mouse line with increased production of A β species, synaptic expression of β -NRXs is decreased. Together, our results indicate that A β Os interact with NRXs and that this interaction disrupts NRX-based synapse organization by destabilizing surface β -NRX on axons.

Results

A candidate screen isolates β -neurexins as A β oligomer-interacting proteins. To test whether there are any synaptic organizers that interact with A β oligomers, we performed cell surface binding assays in which soluble oligomers of amyloid- β (1–42) peptide conjugated with biotin (biotin-A β ₄₂) were added onto COS-7 cells expressing each synaptic organizer. We first confirmed that the biotin-A β ₄₂ peptides were properly oligomerized into low and high molecular weight oligomers by western blot analysis (Fig. 1a) and also that the biotin-A β ₄₂ oligomers bound COS-7 cells expressing the known A β ₄₂ oligomer receptors, the paired immunoglobulin-like receptor B (PirB)⁴⁶ and the cellular prion protein (PrP^C)⁴⁷ (Fig. 1b). We screened a total of 22 synaptic organizers. We found no significant binding of biotin-A β ₄₂ oligomers on COS-7 cells expressing NLG1 or NLG2 (Fig. 1c) although a previous study reported an interaction between A β Os and NLG1⁴⁵. Instead, we detected significant binding of biotin-A β ₄₂ oligomers on COS-7 cells expressing (NRX1 β S4(-)) (Fig. 1c). Interestingly, we did not detect any binding signals on COS-7 cells expressing any of the other synaptic organizers that we tested including type IIa receptor-type protein tyrosine phosphatases (RPTPs: PTP σ , PTP δ , and LAR), LRRTM2, TrkC, and Slitrk family members (Fig. 1c).

We next checked for a biochemical interaction between NRX1 β and A β Os in a pull-down assay using untagged A β ₄₂ oligomers. High-molecular weight A β ₄₂ oligomers and 5-mer A β ₄₂ oligomers, but not A β ₄₂ monomers, were coprecipitated with purified NRX1 β S4(-)-Fc proteins pre-immobilized on Protein G magnetic beads (Fig. 2a). In contrast, either untagged A β ₄₂ oligomers or monomers were not coprecipitated with purified Fc proteins pre-immobilized on Protein G magnetic beads. These results suggest that A β Os can interact directly with the extracellular domain of NRX1 β S4(-). We next determined the binding affinity by saturation analysis in cell-surface binding assays (Fig. 2b,c). The binding of biotin-A β ₄₂ oligomers to NRX1 β S4(-) increased and became saturated with increasing amounts of biotin-A β ₄₂ oligomers. A Scatchard plot of the binding data revealed that the apparent dissociation constant (K_d) value is 183.5 nM monomer equivalent. Thus the interaction between NRX1 β and biotin-A β ₄₂ oligomers is within the typical nanomolar range for biologically significant ligand-receptor interactions. Together, these results indicate that A β oligomers bind directly with nanomolar affinity to NRX1 β .

Neurexins interact with A β oligomers via the N-terminal histidine-rich domain of β -neurexin1/2/3 and an insert at alternative-splicing site 4 of α / β -neurexin1/2.

Given that the NRX family is composed of many different isoforms such as α / β -isoforms and alternative splicing site 4 (S4)-positive or S4-negative isoforms¹⁸, which have differing binding affinity and selectivity for NRX-interacting proteins, we next tested which NRX isoforms interact with A β Os (Fig. 3). In the case of S4-negative isoforms, biotin-A β ₄₂ oligomers interacted with NRX1 β , 2 β and 3 β at a similar level but not with NRX1 α , 2 α or 3 α , indicating that A β ₄₂ oligomers interact with β -NRX-specific domains in the absence of an insert at S4. Given that the N-terminal histidine-rich domain (HRD; amino acids 50–83 in NRX1 β) is unique to the β -isoforms⁴⁸, we next tested the binding of biotin-A β ₄₂ oligomers to COS-7 cells expressing NRX1 β lacking the HRD (HA-NRX1 β Δ HRD) and detected no binding (Fig. 3a,b). Further, COS-7 cells expressing HA-NRX2 β Δ HRD or HA-NRX3 β Δ HRD also displayed no binding signal (Fig. 3a,b). These results indicate that the HRD of β -NRX1/2/3 is one of the domains responsible for A β ₄₂ oligomer binding. Next, we investigated A β ₄₂ oligomer-binding to S4-positive NRX isoforms. Biotin-A β ₄₂ oligomers interacted with S4-positive NRX1 α and 2 α but not 3 α (Fig. 3a,b), indicating that the inserts at the S4 site of NRX1 and NRX2 interact with A β ₄₂ oligomers. Indeed, S4-positive NRX1 β and 2 β displayed stronger binding of A β ₄₂ oligomers than S4-negative NRX1 β and 2 β , respectively (Fig. 3a,b). The enhancement of binding by the S4 insert is similar to the difference in the binding signals of S4-negative and S4-positive NRX1 α and 2 α (Fig. 3b), suggesting that A β ₄₂ oligomer-binding to the S4 insert additively increases the binding of A β ₄₂ to NRX1 β and 2 β . Together, these data indicate that the HRDs of NRX1 β , 2 β and 3 β and the S4 inserts of NRX1 α / β and 2 α / β are responsible for A β ₄₂ oligomer interaction.

A β treatment diminishes neurexin-mediated presynaptic differentiation. NRXs mediate the presynaptic induction activity of NLGs and LRRTM1/2/3 in hippocampal neurons^{18–22,27–30}. We thus tested whether A β ₄₂ oligomers affect NRX-mediated presynaptic differentiation in coculture-based artificial synapse formation assays (Fig. 4). As reported previously, HEK293 (hereinafter HEK) cells expressing NLG1, NLG2, or LRRTM2 induced the accumulation of the excitatory presynaptic marker VGLUT1. NLG1- or NLG2-expressing HEK cells further induced the accumulation of the inhibitory presynaptic marker VGAT in cocultured hippocampal neurons. Treatment with A β ₄₂ oligomers significantly decreased VGLUT1 accumulation induced by NLG1, NLG2, or

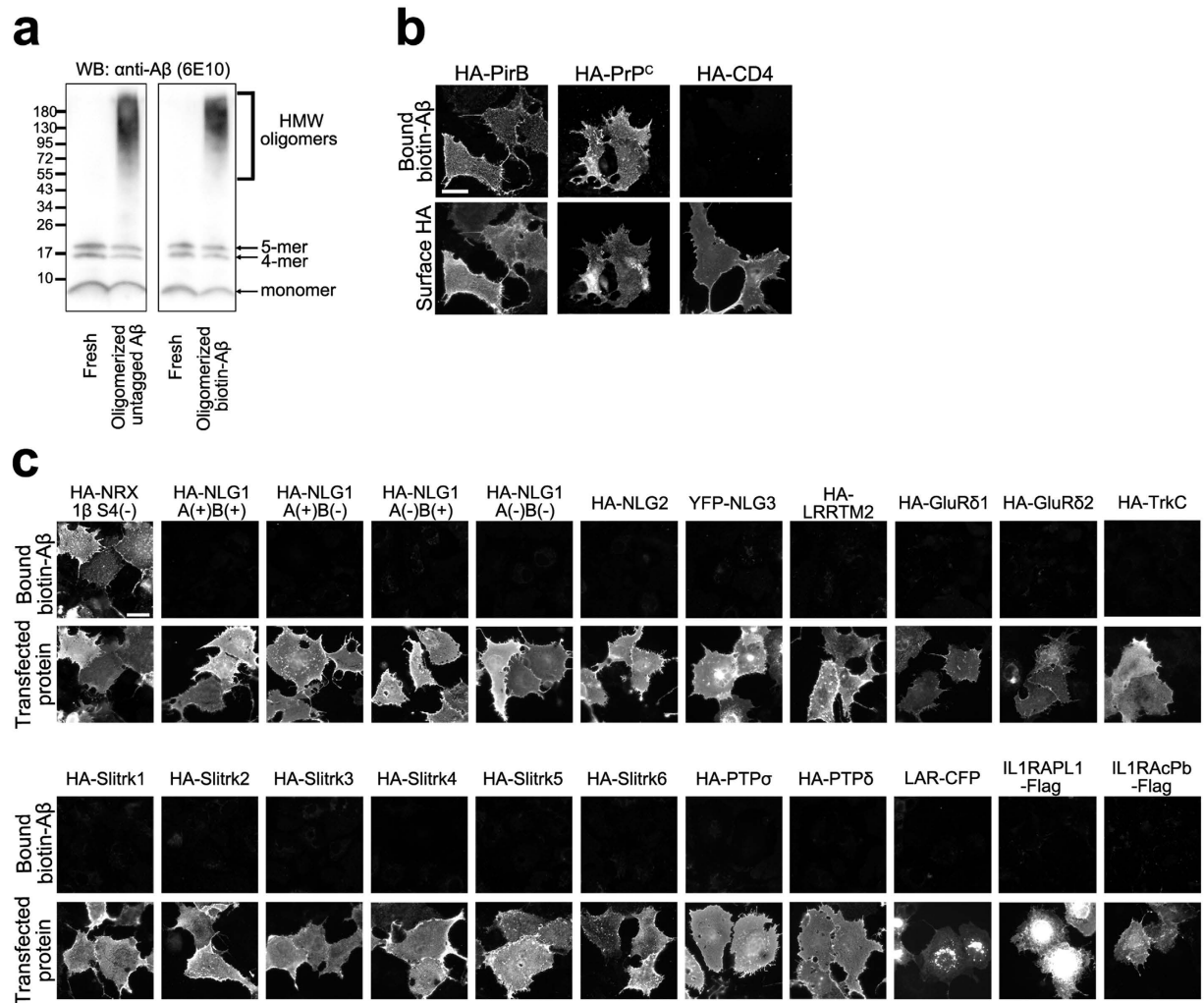


Figure 1. A candidate screen isolates neurexin1 β as an A β_{42} oligomer-interacting protein.

(a) Immunoblotting with an anti- β -Amyloid 1–16 antibody (6E10) confirms the formation of soluble oligomers of untagged amyloid- β (1–42) peptide (A β) and of biotin-tagged A β_{42} peptides (biotin-A β). The preparations include both low and high molecular weight (HMW) oligomers. The preparation without an oligomerization incubation step (Fresh) does not include HMW oligomers. Full gel blots for the cropped blots (a) are shown in the Supplementary Fig. 4. (b) The biotin-A β_{42} oligomers bind to COS-7 cells expressing the N-terminal extracellular HA-tagged known A β_{42} oligomer receptors, paired immunoglobulin-like receptor B (HA-PirB) and prion protein (HA-PrP^c), but not those expressing HA-CD4 as a negative control. Surface HA was immunostained to verify expression of these constructs on the COS-7 cell surface. (c) Representative images showing cell surface binding assays testing for interaction between biotin-A β_{42} oligomers (250 nM, monomer equivalent) and known synaptic organizers. Biotin-A β_{42} oligomers were added to COS-7 cells expressing the indicated construct. Note that biotin-A β_{42} oligomers bind to COS-7 cells expressing HA-neurexin (NRX)1 β S4(–), but not to those expressing any of the other organizers including HA-neuroigin1 (HA-NLG1). For the N-terminal extracellular HA-tagged constructs, surface HA was immunostained to verify expression of the construct on the COS-7 cell surface. Scale bars represent 30 μ m (b,c).

LRRTM2 (Fig. 4a,b). Interestingly, treatment with A β_{42} oligomers had no effect on VGAT accumulation induced by NLG1 or NLG2 (Fig. 4a,c). These data indicate that A β treatment diminishes excitatory, but not inhibitory, presynaptic differentiation induced by NRX-interacting synaptic organizers. Notably, treatment with A β_{42} oligomers did not affect TrkC-induced or Slitrk2-induced VGLUT1 accumulation (Fig. 4a,b), which is mediated by RPTPs in cocultured hippocampal neurons^{22–25}, indicating that A β treatment diminishes NRX-mediated, but not RPTP-mediated, presynaptic differentiation. A β_{42} oligomers did not affect VGAT accumulation induced by HEK cells expressing Slitrk2 (Fig. 4a,c). The observed phenotypes induced by A β_{42} oligomers are not due to the reduction of surface expression levels of the tested organizers on HEK cells because A β_{42} oligomers did not alter their surface expression (Supplementary Fig. 1). Together these results indicate that A β_{42} oligomers selectively diminish NRX-mediated excitatory presynaptic differentiation and also that RPTP-mediated presynaptic differentiation is insensitive to A β_{42} oligomers.

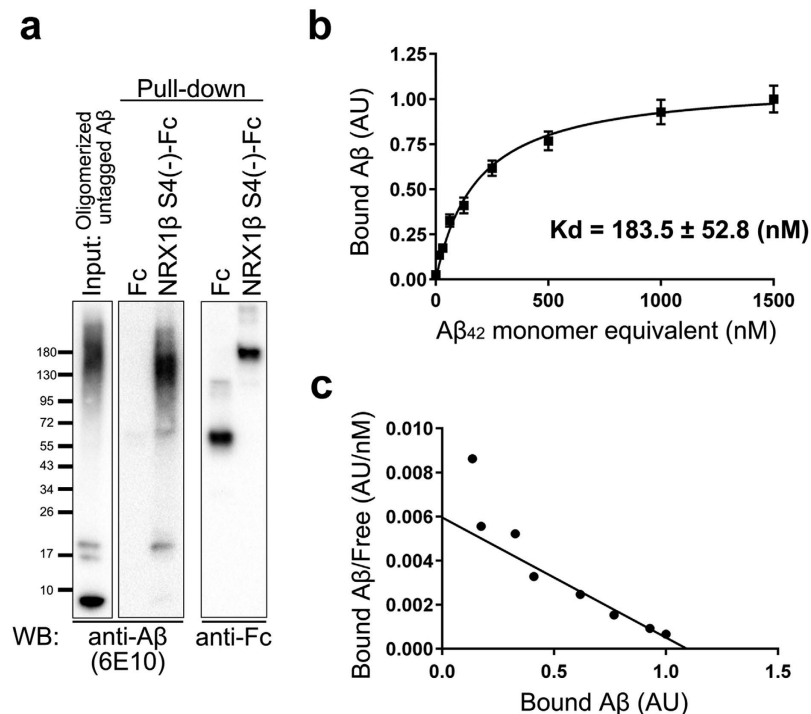


Figure 2. Binding analysis shows that A β_{42} oligomers bind neurexin1 β in the nanomolar range. (a) Pull-down assay of untagged A β_{42} oligomers with purified NRX1 β S4(-)-Fc proteins. Full gel blots for the cropped blots (a) are shown in the Supplementary Fig. 4. (b) Saturable binding of biotin-A β_{42} oligomers to COS-7 cells expressing HA-NRX1 β S4(-). Data are presented as mean \pm SEM. (c) Scatchard plot of binding data shown in (b). The K $_d$ = 183.5 nM monomer equivalent. (n = 30 cells for each plot).

A β treatment has no effect on neurexin interaction with neuroligin1 or LRRTM2. We next investigated cellular mechanisms by which A β_{42} oligomers diminish NRX-mediated presynaptic differentiation. One possibility could be that A β_{42} oligomers interfere with the interaction of NRX with NLG1, NLG2, and/or LRRTM2. To test this, we performed cell surface binding assays using recombinant Fc-tagged NLG1 or LRRTM2 ectodomain proteins (NLG1-Fc or LRRTM2-Fc, respectively). Consistent with previous studies, NLG1-Fc bound to COS-7 cells expressing NRX1 β S4(-) or (NRX1 β S4(+)) (Fig. 5a,b) but not to those expressing a form with a point mutation that completely abolishes NLG interaction, NRX1 β S4(-)D137A⁴⁹ (Fig. 5a,b). LRRTM2-Fc bound to COS-7 cells expressing NRX1 β S4(-), but not NRX1 β S4(+), or NRX1 β S4(-)D137A, as reported previously²⁹ (Fig. 5c,d). Interestingly, the application of biotin-A β_{42} oligomers had no significant effect on the binding of NLG1-Fc or LRRTM2-Fc to any of the NRX1 β constructs (Fig. 5b,d). These data indicate that the diminishment of NRX-mediated presynaptic differentiation by A β_{42} oligomers is not due to A β interference in the interaction of NRXs with NLG1, NLG2, or LRRTM2.

A β treatment decreases surface expression of neurexin1 β on axons. We next tested another possible mechanism: A β_{42} oligomers could decrease the surface expression of NRXs on axons and thereby diminish NRX-mediated presynaptic differentiation. We cotransfected hippocampal neurons with mCherry (for imaging neuronal morphology) and NRX1 β extracellularly tagged with super-ecliptic pHluorin (SEP) (SEP-NRX1 β), and then performed time-lapse imaging of SEP-NRX1 β expressed on mCherry-positive axons (Fig. 6). SEP is a pH-sensitive GFP variant that yields fluorescence at neutral pH (e.g. on the cell surface) but is quenched at low pH (e.g. inside cytoplasmic vesicles), thus it allows for monitoring the surface expression level of tagged proteins⁵⁰. In mCherry-expressing axons, cotransfected SEP-NRX1 β S4(+), SEP-NRX1 β S4(-), or SEP-NRX1 β S4(-) lacking the HRD (SEP-NRX1 β S4(-) Δ HRD) had punctate distributions (Fig. 6a-c). Our immunocytochemistry further confirmed that these SEP-NRX1 β puncta colocalized with VGLUT1 puncta (Supplementary Fig. 2), suggesting that the SEP-NRX1 β proteins likely accumulated at presynaptic boutons. The application of A β_{42} oligomers into the extracellular solution decreased the SEP fluorescent signal of SEP-NRX1 β S4(+) and SEP-NRX1 β S4(-) with similar decay (Fig. 6d). The similarity of the decay suggests that the binding of A β_{42} oligomers to the S4 insert is not essential for A β O-induced reduction of NRX surface expression. On the other hand, the application of A β_{42} oligomers did not affect the SEP signal of SEP-NRX1 β S4(-) Δ HRD (Fig. 6d), which does not bind A β_{42} oligomers (Supplementary Fig. 3). Thus, the binding of A β_{42} oligomers to the HRD is essential for A β O-induced reduction of NRX surface expression. Together with the results of our coculture assays (Fig. 4), these data suggest that A β_{42} oligomers diminish NRX-mediated presynaptic differentiation by decreasing surface expression of β -NRXs on axons.

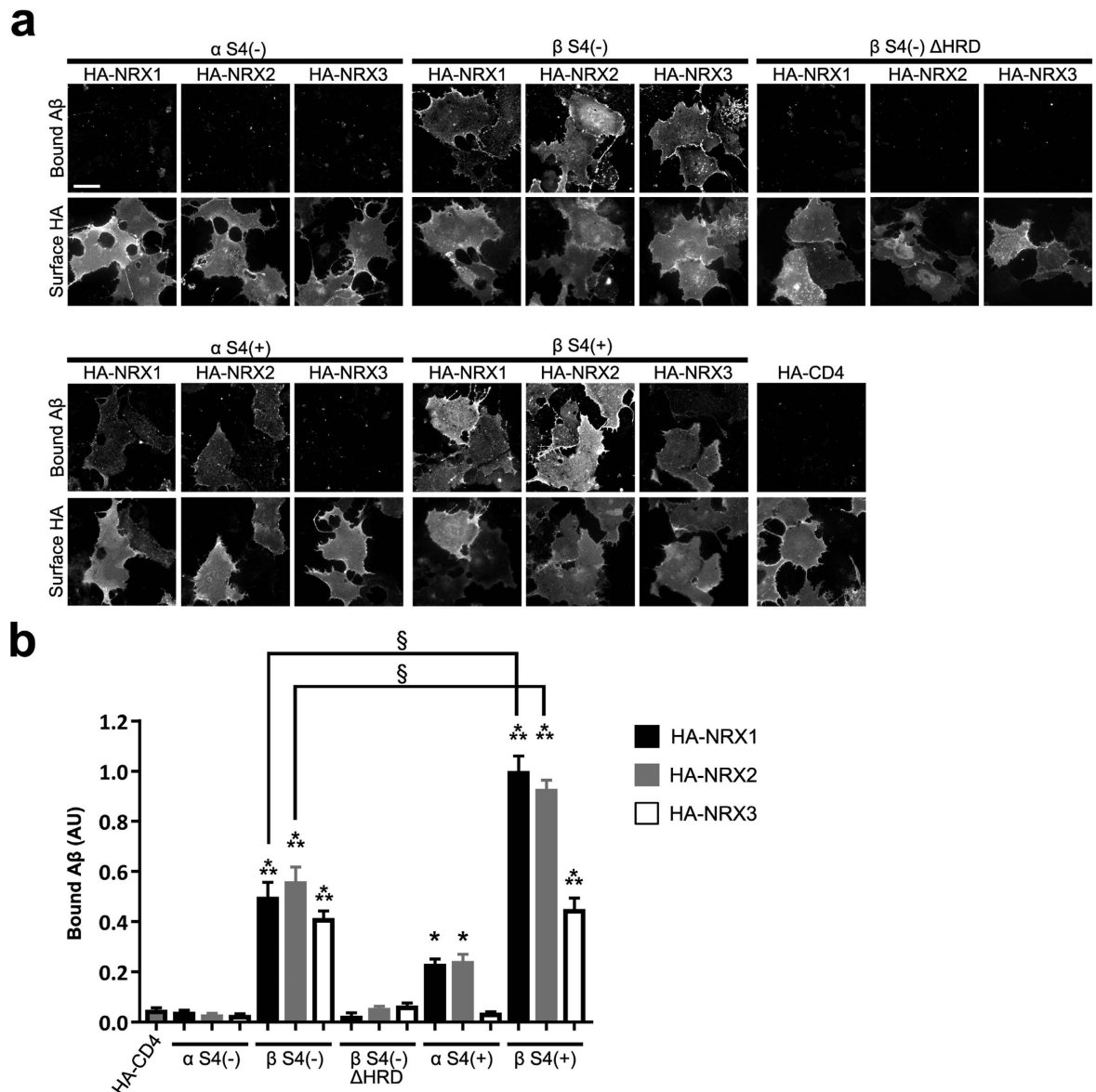


Figure 3. The N-terminal histidine-rich region of β -neurexin1/2/3 and S4 inserts of α / β -neurexin1/2 are responsible for $A\beta_{42}$ oligomer interaction. (a) Representative images showing the binding of biotin- $A\beta_{42}$ oligomers (250 nM, monomer equivalent) to COS-7 cells expressing the indicated isoform of extracellularly HA-tagged NRX. S4(-) and S4(+) indicate without and with an insert at splicing site 4, respectively. HA fluorescent signals correspond to surface HA. Note that in assays with S4-negative isoforms, there is no bound biotin- $A\beta_{42}$ oligomer signal on cells expressing α -NRX1, 2, or 3 whereas cells expressing β -NRX1, 2, and 3 all have significant bound biotin- $A\beta_{42}$ oligomer signal. Cells expressing β -NRX1, 2, and 3 lacking the histidine-rich domain (Δ HRD) have no biotin- $A\beta_{42}$ oligomer binding signal. Cells expressing S4-positive isoforms of α -NRX1 or 2 or β -NRX1, 2, or 3 have significant bound biotin- $A\beta_{42}$ oligomers. Scale bar represents 30 μ m. (b) Quantification of bound biotin- $A\beta_{42}$ oligomers for each NRX construct. $n = 30$ cells for each construct from three independent experiments, one-way ANOVA, $P < 0.001$. * $P < 0.01$ and *** $P < 0.0001$ compared with HA-CD4 and § $P < 0.0001$ between S4(-) and S4(+) by Bonferroni multiple comparisons tests. Data are presented as mean \pm SEM.

Synaptic expression of β -neurexin is decreased in a mouse model of Alzheimer's disease.

Finally, we tested whether $A\beta$ O affect synaptic expression of endogenous NRXs *in vivo* (Fig. 7). We prepared synaptosomal fractions from the hippocampus and the cortex of J20 APP mice, transgenic mice expressing a mutant form of human amyloid precursor protein (APP) that have progressively increasing $A\beta$ O expression and amyloid deposition⁵¹. When compared to those of wild-type littermates, the hippocampal and cortical synaptosomes from J20 APP mice both had significantly reduced levels of β -NRX proteins, but not α -NRX proteins (Fig. 7a,b). These data indicate that there is selective reduction of endogenous β -NRXs in synapses in the J20 transgenic AD mouse model.

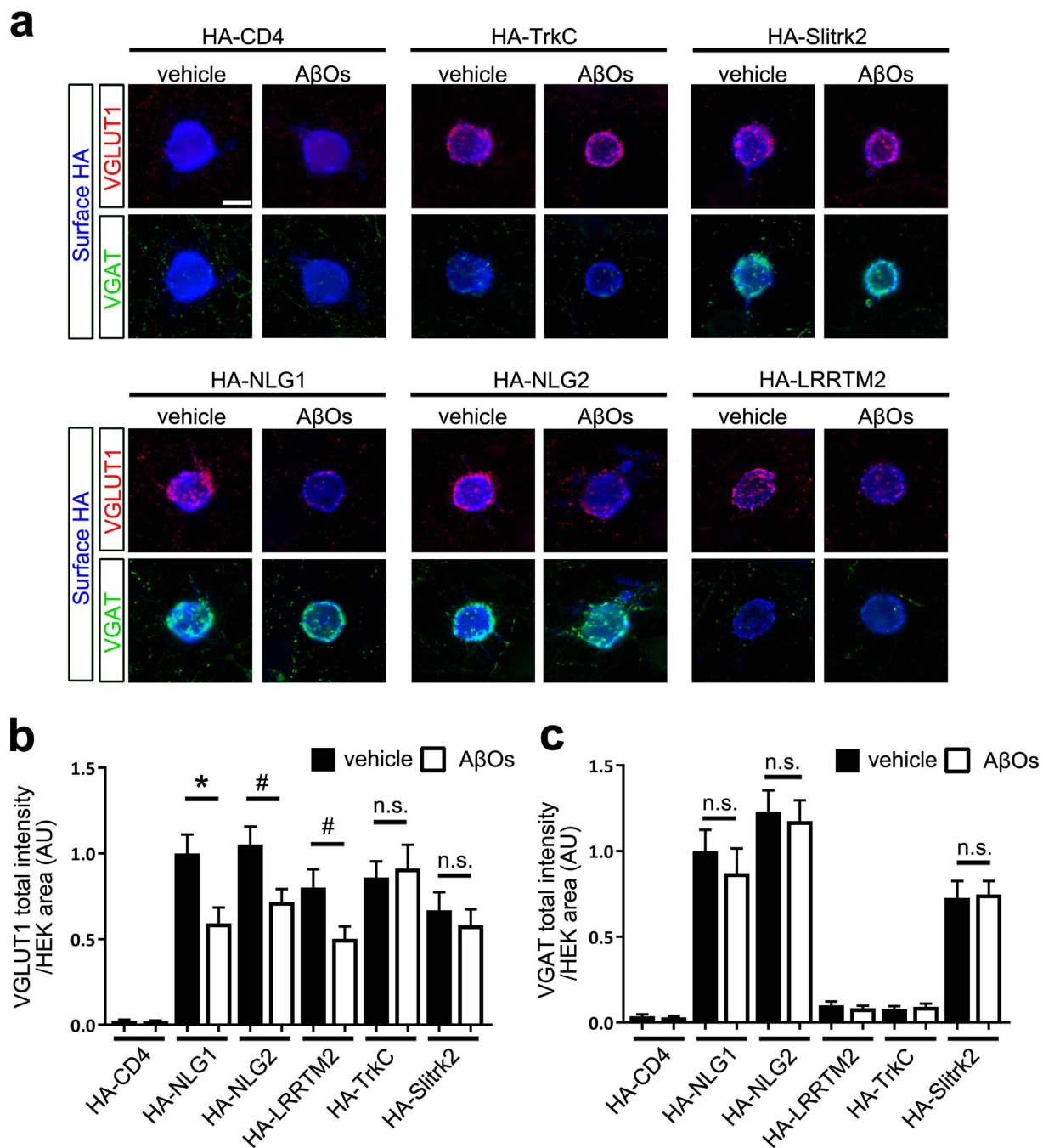


Figure 4. A β_{42} oligomers diminish neurexin-mediated excitatory presynaptic differentiation.

(a) Representative images of triple immunolabeling for VGLUT1, VGAT and surface HA in HEK293 cells expressing the indicated extracellularly HA-tagged construct cocultured with cultured hippocampal neurons and treated with A β_{42} oligomers (A β Os, 500 nM, monomer equivalent) or vehicle. HA-CD4 is used as a negative control protein as it lacks synaptogenic activity. A β Os seem not to affect VGLUT1 accumulation induced by an HA-TrkC non-catalytic isoform (HA-TrkC) or VGLUT1 or VGAT accumulation induced by HA-Slitrk2. In contrast, A β Os seem to decrease VGLUT1 accumulation induced by HA-NLG1, HA-NLG2, or HA-LRRTM2. A β Os seem not to affect VGAT accumulation induced by HA-NLG1 or HA-NLG2. Scale bar represents 20 μ m. (b,c) Quantification of the total intensity of VGLUT1 (b) and VGAT (c) puncta on HEK293 cells expressing the indicated HA-tagged proteins divided by HEK293 cell area. $n = 30$ cells for each construct from three independent experiments, one-way ANOVA, $P < 0.0001$. * $P < 0.05$ and * $P < 0.01$ for the indicated comparisons between vehicle control and A β O treatment by Bonferroni multiple comparisons tests. n.s., not significant. Data are presented as mean \pm SEM.

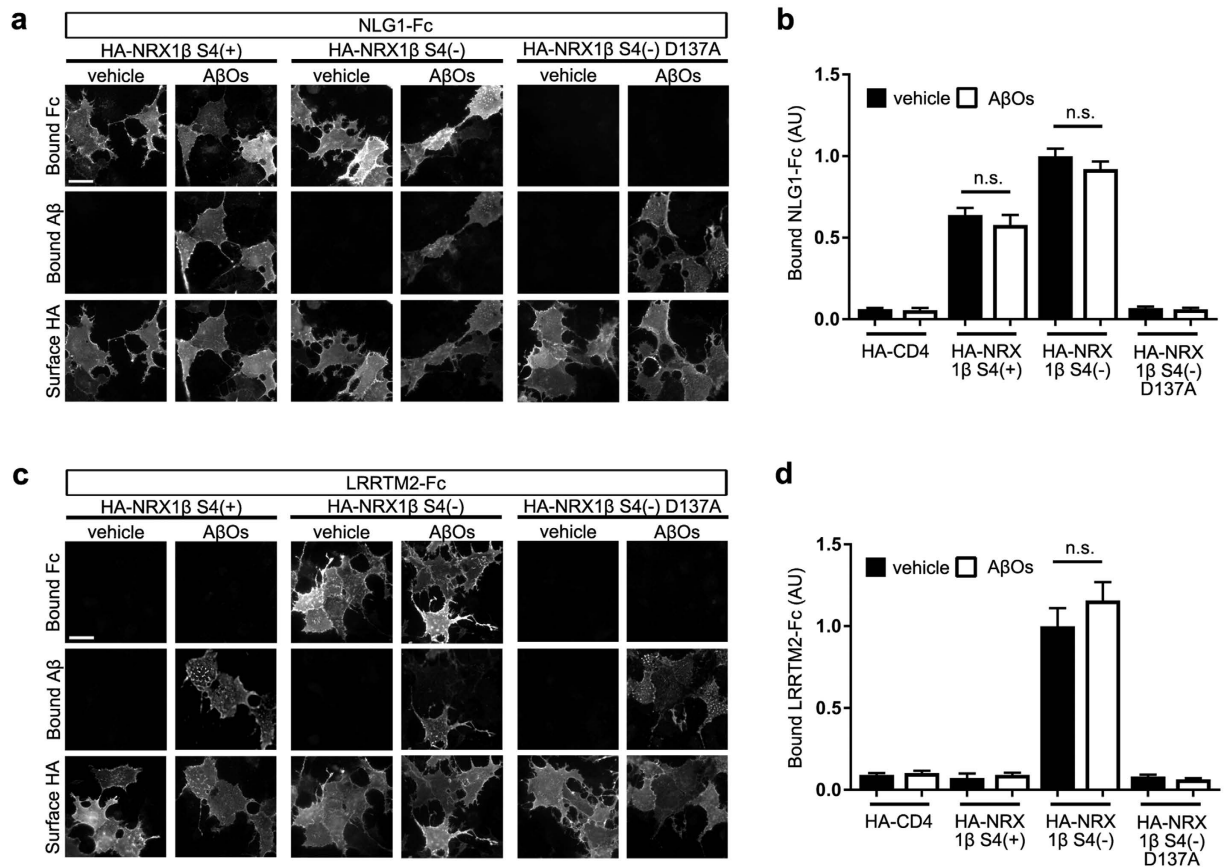


Figure 5. A β_{42} oligomers have no effect on the interaction of neurexin1 β with neuroligin1 or LRRTM2. (a,c) Representative images of triple labeling for bound Fc proteins, bound A β_{42} peptides and surface HA on COS-7 cells expressing the indicated extracellularly HA-tagged neurexin (NRX)1 β construct. Recombinant neuroligin1-Fc (NLG1-Fc; 20 nM) binds to COS-7 cells expressing HA-NRX1 β S4(+) or HA-NRX1 β S4(-) but not to those expressing HA-NRX1 β S4(-)D137A (a), and recombinant LRRTM2-Fc (20 nM) binds to COS-7 cells expressing HA-NRX1 β S4(+) or HA-NRX1 β S4(-)D137A (c). Treatment with A β_{42} oligomers (A β Os, 500 nM, monomer equivalent) does not seem to affect the binding in any of these cases. Scale bars represent 30 μ m. (b,d) Quantification of recombinant NLG1-Fc (b) or LRRTM2-Fc (d) bound to COS-7 cells expressing the indicated HA-NRX1 β constructs treated with vehicle control or 500 nM A β_{42} oligomers. $n = 30$ cells for each condition from three independent experiments, one-way ANOVA, $P < 0.0001$. n.s., not significant by Bonferroni multiple comparisons test. Data are presented as mean \pm SEM.

Discussion

In this study, we uncovered a direct interaction of A β_{42} oligomers with NRXs. We further determined that the HRDs of NRX1 β , 2 β and 3 β and S4 inserts of NRX1 and NRX2 are responsible for the interaction between A β_{42} oligomers and NRXs. A β_{42} oligomers diminish NRX-mediated presynaptic organization by decreasing surface expression of β -NRXs on axons. Further, synaptic expression of endogenous β -NRXs is selectively decreased in a line of transgenic mice with increased production of A β peptides. Together, our findings demonstrate that NRX is an interactor of A β_{42} oligomers and plays a role in A β O-induced synapse pathology.

It has been well known that A β Os have postsynaptic adverse effects such as the inhibition of LTP, the enhancement of LTD and the loss of dendritic spines (see the review of refs 4 and 6 and references therein). These effects are mediated by A β -interacting postsynaptic membrane proteins such as prion^{47,52,53}, PirB⁴⁶ and EphB2⁵⁴, whose binding to A β alters the function of postsynaptic NMDA-type glutamate receptor and/or metabotropic glutamate receptor 5 and consequently affects synaptic plasticity and dendritic spine density. However, A β accumulates at presynaptic terminals as well as at postsynaptic sites, and A β can also distort presynaptic structure and function^{6,9,11}. The mechanisms underlying the presynaptic actions of A β are not well understood. NRXs are presynaptic cell-adhesion molecules crucial for presynaptic organization and functions^{18,19}. Thus the most significant finding of this study is the identification of NRXs as novel A β O-interacting presynaptic membrane proteins. Our data show that A β_{42} oligomers interact with NRXs and that this interaction leads to a decrease in NRX expression on the axon surface. NRXs regulate synapse organization through interacting with multiple postsynaptic adhesion molecules including NLG1-4^{18,19}, LRRTM1/2/3²⁶⁻³⁰, and calyntenin-3³¹. Thus, the NRX family serves as a presynaptic molecular hub to integrate and/or coordinate multiple trans-synaptic organizing signals²². Our findings therefore suggest that A β_{42} oligomers may dampen the presynaptic hub function of NRXs and thereby

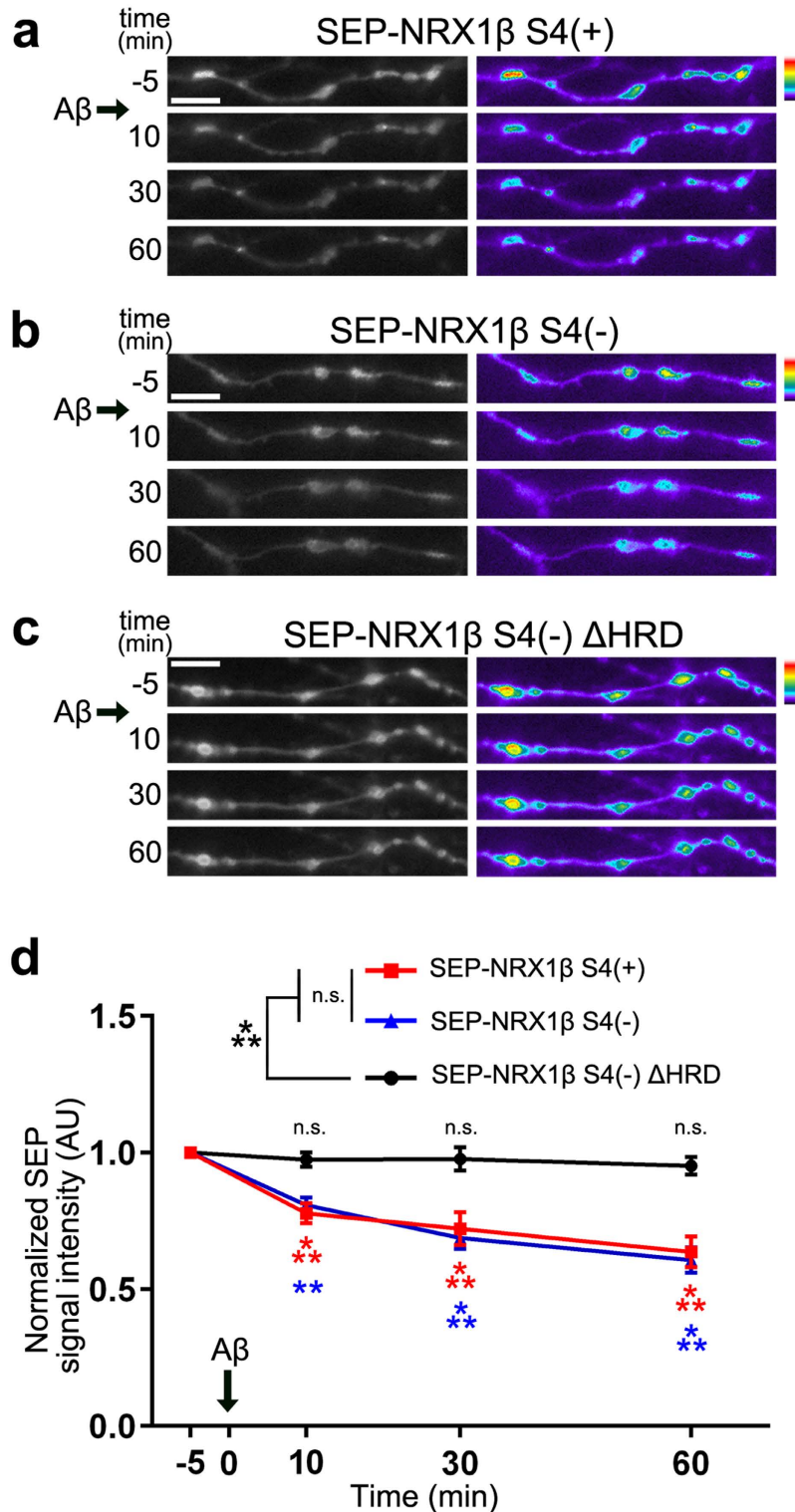


Figure 6. A β_{42} oligomers reduce surface expression of neurexin1 β on axons. (a–c) Representative time-lapse images of axons expressing extracellularly super-ecliptic pHluorin (SEP)-tagged NRX1 β S4(+) (a), SEP-NRX1 β S4(-) (b) and SEP-NRX1 β S4(-) lacking the histidine-rich domain (SEP-NRX1 β S4(-) Δ HRD) (c) treated with A β_{42} oligomers (500 nM, monomer equivalent) at t = 0 min. Scale bars represent 5 μ m. (d) Quantification of SEP intensity signals at 5 min before and 10, 30 and 60 min after A β_{42} oligomer treatment. $n = 29$ for SEP-NRX1 β S4(+), $n = 26$ for SEP-NRX1 β S4(-), and $n = 33$ for SEP-NRX1 β S4(-) Δ HRD puncta from 9 cells for each condition from three independent experiments, two-way repeated measures ANOVA, $F(3, 255) = 42.94$, $P < 0.0001$ for time and $F(2, 85) = 18.95$, $P < 0.0001$ for construct. $**P < 0.001$, and $***P < 0.0001$ compared with SEP signal at 5 min before the treatment, and $***P < 0.0001$ compared with SEP-NRX1 β S4(-) Δ HRD by Bonferroni multiple comparisons tests. n.s., not significant. Data are presented as mean \pm SEM.

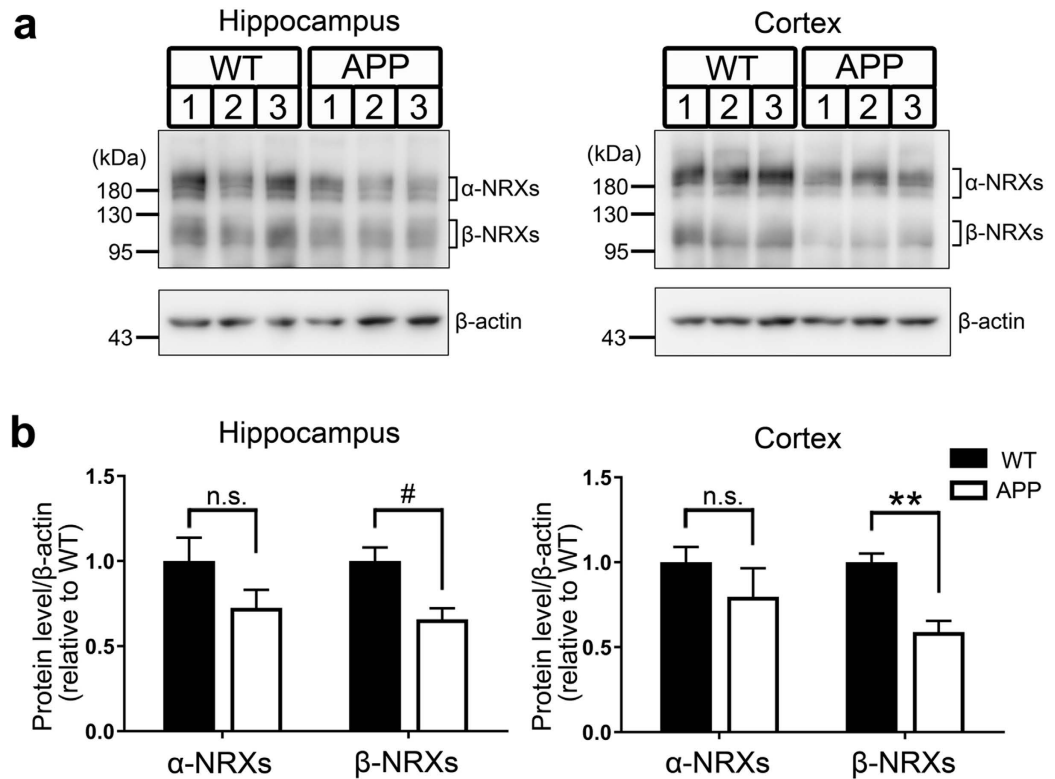


Figure 7. Synaptic expression of endogenous β -neurexins is decreased in J20 APP mice. (a) Representative immunoblots of neurexins (NRXs) in synaptosomes from the hippocampus and from the cerebral cortex of J20 APP mice and wild-type (WT) littermates at 6 months of age. The labels 1, 2, and 3 indicate samples from different mice. Full gel blots for the cropped blots (a) are shown in the Supplementary Fig. 4. (b) Quantification of synaptic expression of β -NRXs (bands indicated by a lower right square bracket in (a)) and α -NRXs (bands indicated by upper right square bracket in (a)) normalized to β -actin protein expression in synaptosomes from the hippocampus and the cortex, expressed relative to WT. $n = 5$ samples per genotype for hippocampus, with each n representing pooled hippocampi from two mice. $n = 6$ samples per genotype for cortex, with each n representing a cortex from one mouse. Unpaired t tests, $^*P < 0.05$ for β -NRXs and $P = 0.15$ for α -NRXs in hippocampus, and $^{**}P < 0.001$ for β -NRXs and $P = 0.31$ for α -NRXs in cortex. n.s., not significant. Data are presented as mean \pm SEM.

disrupt the balance of the multiple synaptic organizing complexes. The dysregulation of NRXs by $A\beta_{42}$ oligomers would therefore be an important mechanism underlying $A\beta$ vulnerability of synapses.

Like members of the NRX family, RPTP family members including $PTP\sigma$, $PTP\delta$, and LAR act as presynaptic hubs by mediating trans-synaptic interactions with multiple organizers such as TrkC, Slitrks, NGL-3, and IL1RAPL1²². Our binding screen shows that RPTP family members do not interact with $A\beta_{42}$ oligomers. Further, our coculture data suggest that $A\beta_{42}$ oligomers diminish NRX-mediated, but not RPTP-mediated, presynaptic differentiation. Thus, NRX-based synaptic organizing complexes are sensitive to $A\beta_{42}$ oligomers whereas RPTP-based complexes are not. Although synapses are vulnerable to $A\beta$, the majority of synapses remain after the treatment of cultured neurons with $A\beta$ O_s^{13,15} and even at the late stage of AD^{3,55}. This suggests that synapses exhibit two conflicting properties: $A\beta$ vulnerability and tolerance. Thus, $A\beta$ vulnerability and tolerance of synapses may be partly determined by two different presynaptic hubs: an $A\beta$ -sensitive hub (NRX) and an $A\beta$ -insensitive hub (RPTP).

In our coculture assays, $A\beta_{42}$ oligomers suppress excitatory presynaptic differentiation induced by HEK cells expressing multiple NRX-interacting synaptic organizer proteins: NLG1, NLG2, and LRRTM2. Our results demonstrating disruption of the hub protein NRX represent one mechanism underlying this effect. Direct interaction of $A\beta$ O_s with these NRX interactors could be another possible mechanism. A previous study reported a direct interaction between $A\beta_{42}$ peptides and the NLG1 ectodomain⁴⁵. However, our cell surface binding assay showed no binding of $A\beta_{42}$ oligomers to NLG1-expressing fibroblasts. This discrepancy could result from a difference in the production of the target proteins: the previous study used soluble recombinant NLG1 ectodomain proteins whereas we used membrane-bound NLG1 expressed on the cell surface. The same previous study⁴⁵ and our binding assay show that NLG2 does not interact with $A\beta_{42}$ oligomers and we also show that they do not bind LRRTM2 in cell surface binding assays. These data support the idea that the molecular mechanism by which $A\beta_{42}$ oligomers suppress the synaptogenic activity of NLG1 and other synaptogenic NRX interactors is by binding and decreasing NRXs on the axon surface, rather than by directly binding multiple synaptogenic NRX interactors.

The effects of A β O may not be limited to synapse organization but may also impact on synaptic function as NRX proteins can regulate neurotransmitter release: α -NRX isoforms regulate Ca²⁺-triggered neurotransmitter release by functionally coupling calcium channels to presynaptic machinery⁵⁶ whereas β -NRX isoforms regulate endocannabinoid-dependent glutamate release probability⁵⁷. A previous study has shown that A β increases the presynaptic release probability of glutamate⁵⁸. Thus, the interaction between NRXs and A β ₄₂ oligomers that we have uncovered here could mediate A β -dependent glutamate release by changing calcium channel activation and/or endocannabinoid signaling pathways. Future studies using α -NRX1/2 double knockout mice and/or β -NRX1/2/3 triple knockout mice could test this possibility.

A β seems to selectively affect glutamatergic (excitatory) presynaptic terminals. A previous study showed that A β has no effect on GABA release probability⁵⁸ and our coculture data show that A β ₄₂ oligomers diminish excitatory, but not inhibitory, presynaptic differentiation induced by NLG1/2. Since our data has also shown that NRX is central to A β vulnerability, the mechanism underlying differential sensitivity of glutamatergic and GABAergic axons to A β likely involves NRX. Our binding assays show that the binding of A β ₄₂ oligomers to NRXs depends on the isoform type (α versus β) and S4 site insertion, and a recent study using single-cell mRNA profiling has shown that there is cell-type-specific expression of NRX isoforms⁵⁹. Thus, comparison of the expression profiles of α - and β -isoforms and S4 splicing in glutamatergic and GABAergic neurons could yield insight into the differing A β sensitivity of their axons.

We have shown here that the molecular mechanism of the A β -NRX interaction involves two domains: the S4 inserts of NRX 1/2 and the HRDs of β -NRX forms. NRX S4 inserts are critical for determining the binding affinity of NLGs with NRXs and the binding selectivity of LRRTMs with NRXs^{18,19,29}. Although A β ₄₂ oligomers bind to the S4 inserts of NRX1 and NRX2, A β ₄₂ oligomers have no effect on the binding of NLG1 or LRRTM2 to NRX1 β S4(+) and also have similar effects on surface expression of SEP-NRX1 β S4(+) and SEP-NRX1 β S4(-). These findings suggest that the binding of A β ₄₂ oligomers to the S4 insert is not essential for A β O-induced diminishment of NRX-mediated presynaptic differentiation. Instead, our binding assays show that the HRDs of NRX1 β , 2 β , and 3 β are necessary for the binding of A β ₄₂ oligomers to β -NRXs and our time-lapse imaging study demonstrates that the HRD of β -NRXs is crucial for A β O-induced reduction of NRX surface expression on axons. The importance of the HRD for axonal expression of NRX is additionally supported by our *in vivo* finding that β -NRXs (which possess an HRD), but not α -NRXs (which lack HRDs), are decreased significantly in synaptosomes of J20 APP mice. Therefore, the HRD likely contributes to the stabilization of surface β -NRXs on axons under normal physiological conditions.

Our binding assays demonstrate that β -NRX HRDs and NRX1/2 S4 inserts are the domains responsible for A β ₄₂ oligomer binding of NRXs. This is helpful to develop new therapeutic strategies aimed at preventing A β -induced synapse pathology, in particular presynaptic dysfunction since NRXs function presynaptically^{18,56,57}. For example, neutralizing antibodies and/or small peptides that block NRX-A β O interactions could normalize presynaptic glutamate release distorted by A β O. Thus, our findings provide new molecular insights into how A β -induced synapse pathology could be prevented and/or reduced.

Materials and Methods

Plasmids. To generate a series of extracellularly HA-tagged neurexin (HA-NRX) constructs, cDNA encoding the mature form of each NRX isoform was subcloned into spNRX1 β -HA-C1, a vector containing a CMV promoter upstream of the N-terminal signal peptide sequence of NRX1 β (spNRX1 β) followed by HA and a multiple cloning site. The following NRX vectors were used as a PCR template for the subcloning: intracellular CFP-tagged mouse NRX1 β S4(+), 1 β S4(-), 1 α S4(+), 1 α S4(-), 2 α S4(+), 2 α S4(-), 3 α S4(+), and 3 α S4(-) (kindly provided by Dr. Ann Marie Craig (University of British Columbia)) and intracellular V5-tagged mouse NRX2 β S4(+), 2 β S4(-), 3 β S4(+), and 3 β S4(-) (kindly provided by Dr. Takeshi Uemura (Shinshu University)). For β -NRX constructs lacking their N-terminal histidine-rich domain (HRD), the coding sequence for the mature forms of NRX1 β lacking HRD (aa 50–83), NRX2 β lacking HRD (aa 54–87) and NRX3 β lacking HRD (aa 48–81) were subcloned into spNRX1 β -HA-C1 following the NRX1 β signal sequence and HA. For extracellularly super-ecliptic pHluorin (SEP)-tagged neurexin1 β (SEP-NRX1 β) constructs, the coding sequence for the mature form of each NRX1 β was subcloned into spNRX1 β -SEP-C1, a vector containing a CMV promoter upstream of spNRX1 β followed by the SEP coding region and a multiple cloning site. All constructs were verified by DNA sequencing. Further details are described in Supplementary Methods.

Animals. All animal experiments were carried out in accordance with the Canadian Council on Animal Care guidelines and approved by the IRCM Animal Care Committee and the McGill University Animal Care Committee. We used heterozygous transgenic adult C57BL/6 mice (6 months old, mixed sex) expressing the human amyloid precursor protein (hAPP) carrying the Swedish (K670N, M671L) and Indiana (V717F) familial AD mutations driven by the platelet-derived growth factor (PDGF) β -chain promoter (APP mice, J20 line)⁵¹ and age-matched wild-type (WT) littermates.

Preparation of A β ₄₂ oligomers. A β (1–42) (r-peptide, A-1002–2, 1 mg) and biotin-tagged A β (1–42) (Anaspec, AS-23523-05, 0.5 mg) were used to generate oligomeric forms essentially as described previously⁶⁰. Full details of the A β ₄₂ oligomer preparation are provided in Supplementary Methods. The preparations were stored at –80°C or used in experiments immediately. Individual A β oligomer stocks were never thawed and re-frozen. To confirm oligomer formation, the preparation was run on a 4–20% TGX precast gel (Biorad) and immunoblotted with anti- β -Amyloid 1–16 (1:5000; mouse IgG1; clone 6E10; Covance).

Neuron culture, coculture-based artificial synapse formation assay and immunocytochemistry. Cultures of rat hippocampal neurons, COS-7 cells, HEK293 cells, coculture-based artificial synapse formation

assays, and immunocytochemistry were performed essentially as reported previously^{23,24}. Transfections into COS-7 and HEK293 cells were performed using TransIT-LT1 (Mirus Bio. LLC). For transfections into hippocampal neurons, the ProFection Mammalian Transfection System (Promega) was used. For artificial synapse formation assays, transfected HEK293 cells were co-cultured with rat hippocampal neurons. Cultures were fixed with paraformaldehyde (4% paraformaldehyde and 4% sucrose in PBS (pH 7.4)) for 12 minutes followed by permeabilization with PBST (PBS + 0.2% Triton X-100). They were incubated with blocking solution (PBS + 3% bovine serum albumin (BSA) and 5% normal goat serum) for 1 hour at room temperature, then with primary antibodies in blocking solution (overnight, 4 °C) and secondary antibodies (1 hour, room temperature). Images were acquired as 12-bit grayscale and prepared using Adobe Photoshop CS5. For quantification, sets of cells were stained simultaneously and imaged with identical settings. Further details are described in Supplementary Methods.

Cell surface binding assay. For testing for binding of biotin- $A\beta_{42}$ oligomers, COS-7 cells on coverslips were transfected with the indicated expression vectors and maintained for 24 hours. The transfected cells were washed with extracellular solution (ECS) containing 168 mM NaCl, 2.4 mM KCl, 20 mM HEPES (pH 7.4), 10 mM D-glucose, 2 mM $CaCl_2$, and 1.3 mM $MgCl_2$ with 100 μ g/ml BSA (ECS/BSA) and then incubated with ECS/BSA containing 250 nM biotin- $A\beta_{42}$ oligomers (monomer equivalent) for 1 hour at 4 °C to prevent endocytosis. The cells were washed in ECS, fixed with paraformaldehyde for 12 min at room temperature, incubated with blocking solution for 1 hour at room temperature, followed by the immunolabeling of surface HA as described above, and then incubated with Alexa594-conjugated streptavidin (1:4000; Jackson ImmunoResearch) and Alexa488-conjugated anti-rabbit IgG (H+L) (1:500; Invitrogen) for 1 hour at room temperature to label bound biotin- $A\beta_{42}$ oligomers and surface HA, respectively. Further details are described in Supplementary Methods.

Pull-down assays. Purified soluble recombinant human NRX1 β S4(–) ectodomain fused to human Fc (NRX1 β S4(–)-Fc, 5268-NX-050, R&D systems) or human Fc (a negative control) generated from the pc4-sp-Fc vector²³ were used for the pull-down assays. NRX1 β -Fc or Fc proteins were pre-immobilized with Protein G magnetic beads (Dynabeads Protein G, Life Technology) in 20 mM sodium phosphate buffer (pH 7.0) for 2 hours at 4 °C. The pre-immobilized NRX1 β -Fc or Fc proteins were then incubated with untagged $A\beta$ oligomers in binding solution (20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, and 1.3 mM $MgCl_2$) for 1 hour at 4 °C. Subsequently, the bead suspensions were washed five times with binding solution. Bound peptides and proteins were eluted with 100 mM glycine-HCl. Eluted samples were diluted in SDS sample buffer without boiling, separated on a 4–20% gradient SDS-PAGE gel and analyzed by western blotting with anti- β -Amyloid 1–16 (1:5000; mouse IgG1; clone 6E10; Covance) or horseradish peroxidase (HRP)-conjugated anti-human Fc (1:10,000; Jackson ImmunoResearch) antibodies.

Time-lapse imaging. For time-lapse imaging, hippocampal neurons cultured on 18-mm coverslips were cotransfected with a SEP-NRX construct and mCherry at 10 days *in vitro* (DIV) and used for imaging at 20–22 DIV. During imaging, the live transfected neurons were mounted in a Chamlide CMB magnetic chamber (Live Cell Instrument) and maintained in ECS at 37 °C controlled by a Tempcontrol 37–2 device (Pecon Germany) without perfusion. $A\beta_{42}$ oligomers (500 nM, monomer equivalent) were manually added into ECS in the chamber 5 minutes after taking the first image. Fluorescent imaging was performed using a Leica DMIRE2 inverted microscope (Leica Germany) equipped with an Orca ER CCD camera (Hamamatsu Japan) and a 63 \times 1.4 NA oil objective lens. All images were acquired by Volocity software (Perkin Elmer) at 1344 \times 1024 resolution with 12 bits/pixel.

Fluorescence quantification. All imaging and image analysis were done while blind to the experimental condition. Analysis was performed by using Metamorph 7.8 software (Molecular Devices), Microsoft Excel, and GraphPad Prism 6. For binding of biotin- $A\beta_{42}$ oligomers and Fc-fusion proteins, the average intensity of bound protein per COS-7 cell area minus off-cell background was normalized to the average intensity of the surface HA signal on COS-7 cells expressing the indicated HA-tagged proteins. For cocultures, fields for imaging were chosen using only the HA and phase contrast channels to locate HA-positive HEK293 cells in neurite-rich regions. The VGLUT1 or VGAT channel was thresholded and the total intensity of the puncta within HA-positive HEK293 cell regions was measured. For time-lapse imaging, the average background intensity of the image before $A\beta$ treatment was measured, and this value was subtracted from the intensity of each frame of the time-lapse image sequences. The axons of transfected neurons were defined based on the morphology of mCherry-expressing neurons. In the image before $A\beta$ treatment, the areas corresponding to puncta of SEP-NRX1 β in mCherry-positive axons were manually traced as regions of interest (ROIs) using Metamorph 7.8. The average intensity of SEP and mCherry signals in these ROIs in each frame was measured. To quantify the effects of $A\beta$ treatment on NRX surface expression, the SEP signal was normalized to the mCherry signal. Correction of the image shift in the x–y plane was done by comparing mCherry and SEP images. Pseudo-color images were created based on the fluorescence intensity range of the image prior to the $A\beta$ treatment by Metamorph 7.8.

Synaptosome preparation and Immunoblotting. Preparation of synaptosome fractions from mice was performed essentially as described previously²⁶. For all samples, protein concentrations were measured in DC Protein Assays (Biorad). After normalizing protein concentration, samples were run on 10% polyacrylamide gels. For immunoblotting NRXs, unboiled samples were used. Signals were developed using Immobilon Western Chemiluminescent HRP Substrate (Millipore) and captured by an ImageQuant LAS 4000 instrument (GE healthcare). Band signal intensity was measured using Metamorph 7.8 software and normalized to β -actin signal intensity for quantification. Further details are described in Supplementary Methods.

Statistical analysis. Statistical tests were performed using GraphPad Prism 6. Data distribution was assumed to be normal. Statistical comparisons were done by Student's unpaired t test, one-way ANOVA and two-way repeated measures ANOVA with *post hoc* Bonferroni multiple comparisons tests, as indicated in the figure legends. All data are represented as the mean \pm standard error of the mean (SEM) from three independent experiments and statistical significance was defined as $P < 0.05$.

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Author Contributions

Y.N. performed a majority of the experiments including cell surface binding screen and assays, pull-down experiments, coculture experiments and time-lapse imaging. Y.T. prepared synaptosomal fractions of J20 APP mice and western blot analysis. A.K.L. performed binding assays. E.H. prepared J20 APP mice for synaptome experiments. H.T. supervised the project. Y.N. and H.T. conceived the project and prepared the manuscript with critical input from E.H. All authors reviewed the manuscript.

Additional Information

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