Interaction of amyloid-β (Aβ) oligomers with neurexin 2α and neuroligin 1 mediates synapse damage and memory loss in mice

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Brain accumulation of the amyloid-β protein (Aβ) and synapse loss are neuropathological hallmarks of Alzheimer disease (AD). Aβ oligomers (AβOs) are synaptotoxins that build up in the brains of patients and are thought to contribute to memory impairment in AD. Thus, identification of novel synaptic components that are targeted by AβOs may contribute to the elucidation of disease-relevant mechanisms. Trans-synaptic interactions between neurexins (Nrxs) and neuroligins (NLs) are essential for synapse structure, stability, and function, and reduced NL levels have been associated recently with AD. Here we investigated whether the interaction of AβOs with Nrxs or NLs mediates synapse damage and cognitive impairment in AD models. We found that AβOs interact with different isoforms of Nrx and NL, including Nrx2α and NL1. Anti-Nrx2α and anti-NL1 antibodies reduced AβO binding to hippocampal neurons and prevented AβO-induced neuronal oxidative stress and synapse loss. Anti-Nrx2α and anti-NL1 antibodies further blocked memory impairment induced by AβOs in mice. The results indicate that Nrx2α and NL1 are targets of AβOs and that prevention of this interaction reduces the deleterious impact of AβOs on synapses and cognition. Identification of Nrx2α and NL1 as synaptic components that interact with AβOs may pave the way for development of novel approaches aimed at halting synapse failure and cognitive loss in AD.

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3 The abbreviations used are: AβO, amyloid-β oligomer; AD, Alzheimer disease; NL, neuroligin; Nrx, neurexin; NOR, novel object recognition; OL, object location; ROS, reactive oxygen species; i.c.v., intracerebroventricular; NMDAR, NMDA receptor; ANOVA, analysis of variance; CM-H2DCFDA, chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate.

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protected against novel object recognition (NOR) memory impairment triggered by AβOs in mice, and anti-Nrx2α further protected against oligomer-induced object location (OL) memory impairment. These findings identify Nrx2α and NL1 as synaptic components targeted by AβOs and indicate that interfering with interactions between AβOs, Nrx2α, and NL1 decreases the impact of AβOs on synapses and cognition. The results may illuminate novel approaches to fight cognitive decline in AD.

Results

AβOs interact with different isoforms of Nrx and NL

We initially screened a commercial phage display peptide library (see “Experimental Procedures”) to identify Aβ-binding peptides presenting sequence homology to synaptic membrane proteins. A systematic search of protein databases in NCBI using BLAST revealed that one of the Aβ-binding peptides thus identified (corresponding to the amino acid sequence IGT-VDRS, henceforth termed IG peptide) presented significant sequence similarity to a sequence motif present in both Nrxα and Nrxβ (Fig. 1A). Interestingly, this motif is contained within the NL1-binding domain in Nrxs (Fig. 1, B and C) (22).

We next aimed to biochemically validate the interaction between AβOs and Nrx. Using a plate binding assay (see “Experimental Procedures”), we first found that AβOs bind to the IG peptide (Fig. 1, D and E). Importantly, AβOs bound to purified recombinant full-length Nrx1α and Nrx2α (but not to BSA, used as a negative control) (Fig. 1, F and G).

Consistent with recent reports (10, 11), AβOs bound purified recombinant NL1 (Fig. 1F). We further found that AβOs robustly bound NL2 but not NL3 (Fig. 1G). These results suggest that different isoforms of Nrxα and NL can be synaptic targets of AβOs.
Neurexin 2α and neuroligin 1 mediate synaptic impact of AβOs

We then sought to investigate whether AβOs interact with human NL1 and Nrx2α. Using a ligand capture assay (see “Experimental Procedures”), we found that AβOs interact with both NL1 and Nrx2α, present in human adult brain tissue homogenates, but not with GABA_2A receptor subunit 5 (GABA_2A R5), mostly expressed at inhibitory synapses), supporting the notion that AβOs target these proteins in the human brain (Fig. 1H). We further confirmed that recombinant NL1 and Nrx2α directly interacted with each other in our plate binding assay (Fig. 1I).

The IG peptide, anti-Nrx2α, and anti-NL1 reduce AβO binding to hippocampal neurons

To determine whether the amino acid sequence motif homologous to Nrx that was identified by phage display was relevant for neuronal targeting by AβOs, we tested the ability of the IG peptide to act as a scavenger for AβOs. AβOs were preincubated in solution with the IG peptide at different molar ratios before addition to neuronal cultures. Hippocampal neurons exposed to AβOs incubated previously at a 5:1 Aβ:IG molar ratio showed a 60% decrease in dendritic AβO immunoreactivity (detected as immunoreactivity toward the NU4 monoclonal antibody), representative images of AβO preparations. Antibodies were added to the cultures 30 min prior to AβOs. Scale bar = 10 μm. J, integrated AβO immunofluorescence levels (NU4 immunoreactivity). Anti-Nrx2α results are from five independent neuronal cultures and AβO preparations (8 μg/ml AβO, n = 3; 16 μg/ml AβO, n = 2). Anti-NL1 (5 μg/ml) results are from three experiments with independent neuronal cultures and AβO preparations. K–N, representative images of AβO binding (LDN1 immunoreactivity) in hippocampal cultures exposed for 3 h to vehicle (P), AβOs (500 nM), AβOs + anti-Nr2α (M), or AβOs + anti-NL1 (N). Antibodies were added to the cultures 30 min prior to AβOs. Scale bar = 10 μm. O, integrated AβO immunofluorescence levels (LDN1 immunoreactivity) in a representative experiment from two experiments that yielded similar results. Error bars correspond to means ± S.D. from three replicates. P–S, representative images of AβO binding (detected by Alexa-streptavidin binding to biotinylated AβOs) in hippocampal cultures exposed for 3 h to vehicle (P), AβOs (Q, 500 nM), AβOs + anti-Nr2α (R), or AβOs + anti-NL1 (S). Antibodies were added to the cultures 30 min prior to AβOs. Scale bar = 10 μm. T, integrated Alexa-streptavidin fluorescence levels in a representative experiment from two experiments that yielded similar results. Error bars correspond to means ± S.D. from three replicates. In all experiments, 20–30 images (from two to three coverslips) were acquired and analyzed per experimental condition per independent culture. Symbols correspond to mean values from each independent experiment. ***, p < 0.001; ****, p < 0.0001, one-way ANOVA followed by Holm-Sidak post test.
Neurexin 2α and neuroligin 1 mediate synaptic impact of AβOs

For this, we used an anti-Nrx2α antibody recognizing an epitope distal to the Nrx–NL interaction domain (Fig. 1C), thus minimizing the chance that the antibody itself might interfere with trans-synaptic Nrx-NL1 interactions. In addition, the anti-NL1 antibody we used recognizes a region distal to the NL1 esterase domain known to bind Nrxs. To exclude the possibility that the antibodies against synaptic proteins could directly recognize AβOs and thus mask their detection, we initially performed a control plate binding assay and found that neither anti-NL1 nor anti-Nrx2α bound to synthetic AβOs (Fig. 2F). Hippocampal neuronal cultures were then incubated for 30 min with anti-Nrx2α or anti-NL1 antibodies and subsequently exposed to AβOs (500 nm for 3 h). Interestingly, both antibodies caused significant decreases in dendritic binding of AβOs, as detected by the immunoreactivities of both NU4 (23) and LDN1 (24) antibodies (Fig. 2, F–J and K–O, respectively).

Detection of neuronal binding of AβOs using biotin–conjugated AβOs and Alexa-conjugated streptavidin yielded similar results (Fig. 2, P–T). Collectively, these observations suggest that both Nrx2α and NL1 are involved in dendritic binding of AβOs in hippocampal neurons.

Anti-Nrx2α and anti-NL1 prevent AβO-induced neuronal oxidative stress and loss of dendritic spines and synapses

AβOs trigger neuronal oxidative stress (25–27), as confirmed here with cultured hippocampal neurons (Fig. 3). When neuronal cultures were pretreated with antibodies against Nrx2α and NL1, AβOs failed to induce excessive generation of reactive oxygen species (ROS) (Fig. 3).

AβOs have been shown to cause dendritic spine elimination and synapse loss in vitro (8, 28–30), in vivo (29, 31), and in ex vivo human brain slices (32). We hypothesized that interactions of AβOs with Nrx/NL1 could mediate spine/synapse loss in cultured hippocampal neurons. To test this hypothesis, we investigated whether anti-Nrx2α or anti-NL1 antibodies protected synapses from the toxic impact of AβOs.

We initially assessed dendritic spine density in hippocampal cultures by phalloidin labeling. In line with previous studies (8, 28), exposure of hippocampal neurons to AβOs for 24 h reduced the density of dendritic spines. Interestingly, pretreatment of cells with anti-NL1 (Fig. 4, A–D) or anti-Nrx2α (Fig. 4, E–H) prevented oligomer-induced loss of dendritic spines.

Further, pretreatment of hippocampal cultures with anti-NL1 blocked AβO-induced loss of synaptophysin/PSD-95 co-localized puncta, a readout of synapse density in cultured neurons (33) (Fig. 5, A–D). Similarly, anti-Nrx2α prevented synapse loss induced by AβOs in hippocampal neurons, as measured by synaptotagmin/PSD-95 co-localization (Fig. 3, E–H). Collectively, the results indicate that interaction of AβOs with NL1 and Nrx2α contributes to AβO-induced neuronal oxidative stress and synapse loss.

Anti-Nrx2α and anti-NL1 prevent AβO-induced memory impairment in mice

Last, we asked whether antibodies targeting Nrx2α/NL1 could prevent memory impairment induced by intracerebroventricular (i.c.v.) infusion of AβOs in mice. Initial analysis in an open field arena showed that the exploratory behavior and locomotor activity of mice were not affected by treatments in any of the experimental groups (data not shown). In line with our previous reports (29, 31, 34), mice infused i.c.v. with a single dose of 10 pmol AβOs showed impaired NOR memory 24 h post-infusion (Fig. 6, A–C). Interestingly, i.c.v. infusion of anti-Nrx2α or anti-NL1 (30 min prior to AβOs) prevented NOR memory impairment induced by AβOs (Fig. 6). Control experiments showed that an unrelated IgG (anti-GABA,A5) had no effect on cognitive impairment induced by AβOs (Fig. 6B).

We further investigated whether anti-Nrx2α and anti-NL1 could prevent AβO-induced memory impairment in the OL task (Fig. 6, D and E), which assesses a contextual/location type of memory that is more dependent on the hippocampus than NOR (35, 36). We found that, although AβO-injected mice failed to identify the displaced object, mice injected with anti-Nrx2α had preserved OL memory (Fig. 6E). There was also a trend toward prevention against cognitive impairment in mice injected with anti-NL1, but this was not statistically significant (Fig. 6E).

Figure 3. Anti-Nrx2α and anti-NL1 prevent AβO-induced neuronal oxidative stress. A–G, representative DCF fluorescence images from hippocampal neurons exposed for 4 h to vehicle (A and E), AβOs (B and F, 500 nm), AβOs + anti-NL1 (C), or AβOs + anti-Nrx2α (G). Scale bars = 60 μm. D and H, integrated DCF fluorescence (normalized by cell number in each image, as determined from bright-field images of the same fields) obtained from five (for anti-NL1) or three (for anti-Nrx2α) independent experiments with different hippocampal cultures and AβO preparations. Fluorescence was quantified using ImageJ. Symbols correspond to mean values from each independent experiment. *p < 0.05 compared with vehicle, ANOVA followed by Dunnett post test. Veh, vehicle.
neuronal cultures and AβO preparations (20 fields/experimental condition; 3 dendritic segments/field). Symbols correspond to mean values from each independent experiment. *, p < 0.05; **, p < 0.001, one-way ANOVA followed by Holm-Sidak post test. Veh, vehicle.

Discussion

Synapse loss is a cardinal feature of AD and is considered the best neuropathological correlate of memory impairment (1, 2). A large body of evidence indicates that synapses are targeted by AβOs and become dysfunctional in AD (reviewed in Refs. 3, 5–7, 37). Thus, approaches aimed to identify specific synaptic components targeted by Aβ hold significant potential to illuminate novel therapeutic strategies in AD. Using phage display of a peptide library, we identified an Aβ-binding heptapeptide that was highly homologous to a sequence motif present in the NL-binding site in Nrxs and represents a potential novel Aβ binding site at synapses.

Nrxs and NLs are cell adhesion molecules that promote synapse stabilization and plasticity, learning, and memory (15, 18). Dysfunction in Nrx-NL interactions and related signaling pathways has been implicated in a variety of neurological disorders (reviewed in Ref. 18), including AD. Indeed, a loss-of-function mutation in NL1 has been associated with increased risk of AD (13), and epigenetic suppression of NL1 has been reported to mediate Aβ-induced neurotoxicity (14).

Here we report that AβOs bind Nrx1α and Nrx2α as well as NL1 and NL2. Our findings further indicate that AβOs interact with Nrx through its NL-binding domain. This raises the possibility that AβOs could compromise the Nrx-NL interaction, thus contributing to synapse destabilization and failure. This is supported by our findings that antibodies targeting Nrx2α and NL1 prevented AβO binding and oligomer-induced spine/synapse loss in hippocampal neurons, establishing these proteins as mediators of AβO interactions at synaptic terminals.

In the experiments employing the IG peptide, we modified the original sequence of the peptide identified by phage display to make it identical to the homologous amino acid sequence motif present in Nrxα. Two modifications were included: replacing a positively charged arginine residue with a neutral isoleucine residue at position 6 of the sequence and introducing an additional isoleucine residue at the C terminus of the pep-
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A

B

C

D

E

Figure 6. Anti-Nrx2α and anti-NL1 prevent memory impairment induced by AβOs in mice. A–E, male Swiss mice received one i.c.v. infusion of 1200 ng of anti-Nrx2α, 800 ng of anti-GABAAR subunit 5 (used as a negative control and indicated as IgG), or 750 ng of anti-NL1 30 min prior to i.c.v. infusion of AβOs (10 pmol), as indicated. Mice were tested in the NOR paradigm 24 h after infusion of AβOs (A–C) or in the OL test 8 days after infusion of AβOs (D and E). A and D show schematics illustrating how the NOR and OL tests are performed, respectively. B and C, the percentage of time spent exploring a familiar (old) or novel (new) object used in the test session of the NOR test. E, the percentage of time spent exploring the non-relocated object in the familiar location or the relocated object in the novel location in the OL test. Error bars represent means ± S.E., and symbols represent individual mice in each experimental group. *, p < 0.05, one-sample Student’s t test comparing the mean value for the novel object with the fixed value of 50%. Veh, vehicle.

tide. The former amino acid replacement (arginine to isoleucine) changes the overall charge of the peptide from neutral to negative at physiological pH values, which might have had an impact on the interaction of the IG peptide with Aβ. However, our in vitro binding results revealed that this was not the case, suggesting that the interaction between the IG peptide and Aβ was not much affected by changes in peptide electrostatics. Introduction of the C-terminal isoleucine residue in the IG peptide, mimicking the amino acid sequence in Nrxα, may have been beneficial in terms of the establishment of non-polar interactions with Aβ.

Although NLs and Nrxs were originally described as structural proteins, they have been linked to mechanisms that control long-term potentiation and memory consolidation (38–40). Nrx-NL interactions and trans-synaptic signaling regulate synapse stability and NMDAR function in the postsynaptic membrane (41, 42). Mounting evidence suggests that NMDAR-dependent calcium influx is germane to Aβ neurotoxicity (3, 26, 27, 43, 44). A particular outcome of excessive calcium influx into neurons is an abnormal increase in ROS (27, 45). Indeed, brain oxidative stress is a hallmark of AD pathology (46–48). It is mostly expressed at inhibitory synapses (9, 26, 52, 53), as NL1 is mostly expressed in excitatory terminals (12). A number of molecules present at excitatory synapses have been described to mediate AβO neurotoxicity, and we now provide evidence supporting the notion that interference with trans-synaptic interactions between Nrx and NL by AβOs could contribute to synaptic and memory impairments in AD.

Intriguingly, and in contrast with previous findings (10), our results revealed a robust interaction between AβOs and NL2, which is mostly expressed at inhibitory synapses. Nonetheless, the predominance of excitatory terminals as AβO targets (8), it remains to be determined whether AβO-NL2 interactions are as relevant as AβO-NL1 interactions to explain synapse failure and memory outcomes in AD.

Our finding that AβOs bind to Nrx to promote synaptotoxicity in cultured neurons extends the impact of AβOs to an essential presynaptic element. In addition to postsynaptic defects and synapse loss, it seems plausible that this interaction contributes to the presynaptic dysfunction induced by AβOs (3).
We have demonstrated previously that a single i.c.v. injection of AβOs (10 pmol) causes memory impairment in NOR and contextual fear conditioning in mice (29, 31, 34, 54). Our results demonstrated that anti-Nrx2α prevented AβO-induced impairment in both object recognition (NOR) and contextual/spatial (OL) forms of memory. On the other hand, anti-NL1 prevented AβO-induced memory deficits in the NOR paradigm but not in the OL test. NOR memory appears to rely largely on frontal and perirhinal cortical circuits, whereas OL is predominantly dependent on the hippocampus (35). Our findings thus suggest an important role for AβO-Nrx interactions in impairing both recognition and contextual memory processes that are dependent on hippocampal and cortical circuits. In contrast, the selective protective effect of anti-NL1 on NOR memory suggests that AβO-NL interactions may play a more relevant role in disrupting object recognition than contextual/location memory.

We further attempted to determine the protective actions of anti-Nrx2α and anti-NL1 in the Morris water maze, an established hippocampal spatial memory paradigm (55), as well as in a modified water maze protocol, the so-called memory flexibility test, originally developed to detect age-related memory deficits in a transgenic mouse model of AD (56). However, we found that a single i.c.v. injection of AβOs had no impact on spatial memory in either task (data not shown). This may be related to the fact that water maze paradigms employ multiple training sessions (performed during several days) in an aversive environment. This could lead to stronger memory formation, not likely to be affected by a single infusion of a low dose of oligomers. Conversely, NOR and OL are grounded by innate curiosity and voluntary exploratory behavior toward novelty after a single training session in a non-aversive environment. Memories established in a single event are perhaps more vulnerable than ones established over multiple training sessions (57), which may explain why NOR and OL are more sensitive to AβO-induced memory impairment.

Our findings that Nrx2α and NL1 present in human cortical homogenates bind AβOs and that antibodies against Nrx2α and NL1 protected mice from cognitive impairment triggered by AβOs suggest that the impact of AβOs on Nrxs and NL1 could have potential implications for translational approaches in humans. However, further studies are required to determine the specific mechanisms underlying the preservation of cognitive function by anti-Nrx2α and anti-NL1. Antibodies targeting sites distal from the Nrx/NL-interacting domain in each protein (as done here) may sterically hinder the interaction of AβOs with Nrxs/NLs, leading to preservation of normal synaptic signaling and function.

In conclusion, we provide biochemical and cell biology evidence that AβOs bind presynaptic Nrx at or near the site of Nrx-NL1 interaction and that this instigates synapse damage and neuronal toxicity. Significantly, our findings indicate that AβO-binding to Nrx2α or NL1 mediates the impact of AβOs on synapses and memory in mice. Taken together, the results identify the trans-synaptic partners Nrx and NL as novel AβO targets and suggest that interfering with AβO binding to Nrx-NL could constitute a novel approach toward much-needed AD therapeutics.

### Experimental procedures

**Ethics statement**

All procedures were in accordance with the Principles of Laboratory Animal Care (National Institutes of Health) and performed in certified facilities under protocols approved by the Institution Animal Care and Use Committee (IACUC) of the Federal University of Rio de Janeiro (protocols IBQM 022 and IBQM 044). Experiments involving human cortical tissue were approved by the Committee for Research Ethics of the Clementino Fraga Filho University Hospital of the Federal University of Rio de Janeiro (protocol 0069.0197.000-05). Donors gave written informed consent for the use of brain tissue that would otherwise have been discarded.

**Reagents**

Synthetic Aβ1-42 was from American Peptide (Sunnyvale, CA). Aβ1-40, used exclusively for the biopanning experiment (see below), was from Bachem (Torrance, CA). Culture media/reagents, Alexa-labeled secondary antibodies, CM-H₂DCFDA, and ProLong Antifade reagent were from Invitrogen. Antibodies against Nrx2α (ab34245), NL1 (ab56882), and GABA_A_R5 (ab83003) were from Abcam (Cambridge, MA). Anti-synaptotagmin (105311) was from Synaptic Systems (Göttingen, Germany). Anti-synaptophysin was from EMD Millipore (Billerica, MA). Anti-PSD95 was from Cell Signaling Technology (Danvers, MA). The IGTVDISI peptide (see below) was synthesized by EZ Biolab (Carmel, IN) with ≥98% purity. Purified recombinant Nrx1α, Nrx2α, NL1, NL2, and NL3 were from R&D Systems (Minneapolis, MN). The NU4 monoclonal antibody (23) was a kind gift from Dr. William L. Klein (Northwestern University). Rabbit polyclonal anti-AβO (LDN1) was generated in our laboratory by active immunization of rabbits with synthetic AβO preparations and recognizes both low- and high-molecular-weight Aβ oligomers but not monomers (24).

**Aβ preparation and characterization for the biopanning experiment**

Aβ1-40 was diluted to 0.1 μg/ml in 50% trilfluoroethanol/PBS, and 50-μl aliquots were dried in the wells of a 96-well plate at 37 °C under constant shaking for 16 h. Size-exclusion chromatography-HPLC (using the intrinsic fluorescence emission of the single tyrosine residue in Aβ) showed that Aβ1-40 solubilized in 50% trilfluoroethanol/PBS solution displayed no sign of aggregation or oligomerization for at least 24 h (data not shown).

**AβO preparation and characterization**

AβOs were prepared weekly from synthetic Aβ1-42 following the original procedure described by Lamb et al. (58) and were routinely characterized by SEC-HPLC as described previously (24, 27, 31, 32). For biotinylated AβO preparations, a 1:4 ratio of biotinylated:non-biotinylated Aβ1-42 was used. Oligomers were maintained at 4 °C at all times and used within 48 h of preparation.

**Primary hippocampal neuronal cultures**

Cultures were prepared as described previously (24, 28, 29, 59). Briefly, 18-day-old embryos were obtained from Wistar.
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rats. Hippocampi were dissected, digested in trypsin, and plated onto poly-L-lysine-treated coverslips. Cultures were maintained in Neurobasal supplemented with B-27, penicillin/streptomycin, and amphotericin B for 18–21 days at 37 °C in a humidified atmosphere of 5% CO₂.

Biopanning against Aβ

Screening of a phage display peptide library for Aβ-binding peptides was performed as described previously (60–62). Briefly, wells coated previously with non-aggregated Aβ1-40 (prepared as described above) were blocked in 0.5% bovine serum albumin in PBS for 1 h, and 10 μl of a commercially available phage display library (PhD-C7C, New England Biolabs, Ipswich, MA) was added to each well. The procedures were carried out according to the instructions of the manufacturer. Among 24 different phages bound to Aβ, one expressed the amino acid sequence IGTVDRS displayed on the phage filamentous protein. To identify candidate proteins to which this amino acid sequence might belong, the sequence was compared with protein sequences deposited in several data banks (available at NCBI) using BLAST.

In vitro plate binding assay

The IGTVDISI peptide (referred to as IG peptide, comprising a slight amino acid sequence modification relative to the original IGTVDRS sequence identified by phage display to correspond exactly to the amino acid sequence present in Nrxα), purified recombinant Nrx1α, Nrx2α, NL1, NL2, and NL3 were freshly resuspended in PBS and added to the wells of a 96-well plate (0.5 μg/well for Nrxs, 1 μg/well for NLs), and allowed to dry overnight at 37 °C. AβOs or vehicle was then added to each well and incubated overnight at 4 °C under gentle agitation. Wells were washed with PBS, blocked for 2 h with 1% BSA in PBS, washed six more times in PBS, and incubated for 1 h with 0.3 μg/ml anti-AβO NU4 monoclonal antibody (23). Wells were incubated with HRP-conjugated secondary antibody for 1 h, developed with SuperSignal Femto substrate, and imaged on photographic film. Control wells were coated with BSA. Luminescence values for NU4 immunoreactivity were quantified using NIH ImageJ software (63).

Immunocytochemistry

Hippocampal cultures were fixed with 4% paraformaldehyde containing 4% sucrose for 10 min and blocked for 1 h with 10% normal goat serum in PBS. For AβO binding assays in hippocampal neurons, cells were incubated overnight at 4 °C with 1 μg/ml monoclonal NU4 antibody (23) or 1 μg/ml polyclonal LDN1 antibody (24) in 10% NGS under non-permeabilizing conditions. After washing, Alexa-labeled anti-mouse or anti-rabbit IgG (1:2000 in 1% NGS in PBS) was added.

For biotinylated AβO assays, cells were incubated with Alexa 566-conjugated streptavidin (1:2000 in 10% NGS). After 2-h incubation at room temperature, cells were extensively washed in PBS and mounted in ProLong Antifade Gold with DAPI. Images were obtained from 10–15 randomly chosen fields per experimental condition, from two to three coverslips under each condition, using a ×63 objective in a Zeiss Axioplan microscope. Integrated fluorescence intensity was measured using ImageJ software. For synapse density assays, we used a slightly modified protocol that included cell permeabilization with 0.1% Triton X-100 for 5 min prior to antibody incubation. Mouse anti-synaptophysin (1:500), mouse anti-synaptotagmin (1:1000), or rabbit anti-PSD-95 (1:1000) primary antibodies were incubated for 2 h at room temperature. Alexa Fluor 488-conjugated goat anti-rabbit (1:300) and Alexa Fluor 546-conjugated goat anti-mouse (1:1000) secondary antibodies were incubated for 1 h at room temperature, and cells were imaged.

To determine co-localization of synaptic proteins, neurons separated by a distance of at least two cell body diameters from neighboring cells were selected. Green and red channel images were merged, and co-localization was quantified using the Puncta Analyzer plug-in in ImageJ as described previously (29, 33, 64). In each experiment, at least 30–40 neurons were analyzed per experimental condition (with two to three coverslips per condition).

Phalloidin labeling

For dendritic spine labeling, cells were fixed in freshly prepared 4% formaldehyde solution for 15 min, permeabilized in 0.1% Triton X-100 for 5 min, blocked in BSA 3% for 60 min and labeled with Alexa Fluor 594-phalloidin for 2 h, according to the instructions of the manufacturer, as described previously (28). Coverslips were DAPI-counterstained and mounted on ProLong Antifade Gold reagent for imaging.

ROS

ROS formation was evaluated in living neurons using CM-H2DCFDA as described previously (25–27). Hippocampal cultures were incubated for 4 h at 37 °C with vehicle or 500 nM AβOs in the absence or presence of anti-NL1 or anti-Nrx2α antibodies. When present, antibodies were added to the medium 30 min before AβOs. 2 μM CM-H2DCFDA was loaded during the last 40 min of incubation with AβOs. Neurons were rinsed three times in warm PBS containing 2% glucose and immediately imaged on a Nikon Eclipse TE300 inverted microscope. Analysis of DCF fluorescence was carried out using ImageJ software. Twelve images were acquired under each experimental condition, carried out in triplicate (~300 cells analyzed per condition). Three and four independent experiments (with different hippocampal cultures and AβO preparations) were performed using anti-Nrx2α and anti-NL1, respectively.

Ligand capture assay in human cortical tissue

Healthy cortical tissue was obtained from patients with drug-resistant temporal lobe epilepsy subjected to surgical interventions for removal of epileptic foci, as described previously (32). Tissue fragments were immediately frozen in liquid nitrogen until the assay. Tissue was homogenized in radioimmune precipitation assay buffer (5 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, and 1% Triton X-100) containing protease and phosphatase inhibitor cocktails (Thermo-Pierce), and total protein concentration was determined by BCA. 500 μg of total protein was added to AβO-coated wells (1 μg AβO/well) and incubated overnight at 4 °C. BSA (1%) was used as a control. Wells were
Intracerebroventricular infusions in mice

We used 2- to 3-month-old male Swiss mice obtained from an animal facility at the Federal University of Rio de Janeiro. Intracerebroventricular administration of AβOs (10 pmol), anti-NL1 (750 ng), or anti-Nrx2α (1200 ng) was performed (a single dose delivered in 1.5 μl per animal) as described previously (29, 31, 34). Briefly, animals were anesthetized using 2.5% isoflurane through a vaporizer system and gently restrained only during the infusion procedure (~5 min). A 2.5-mm-long needle was inserted in the left hemisphere according to the following coordinates: 1 mm to the left of the midline point equidistant from each eye and 1 mm posterior to a line drawn through the anterior base of the eye. When used, anti-NL1 or anti-Nrx2α was infused 30 min prior to AβOs.

NOR test

NOR was used to evaluate short-term declarative memory in mice. In this test, mice are exposed to a known object (presented previously during a training phase) and a novel object (distinct in shape, color, and size and presented simultaneously with the known object during a test phase). Mice with preserved memory spend more time exploring the novel object than the familiar (previously presented) object, therefore indicating they remember the object being presented before. NOR was carried out in an open field arena measuring 30 × 30 × 45 cm. Before training, each animal was subjected to a 5-min-long habituation session. Training consisted of a 5-min-long session during which animals were exposed to two identical objects. The amount of time mice spent exploring each object was recorded. Two hours later, one of the objects was replaced by a novel object, and the animals were again placed in the arena for the test session. The time spent exploring each object during the test session and analyzed using one-sample Student’s t test, comparing the mean exploration time for each object with the chance value of 50%. Animals that recognize the relocated object as such explore it for more than 50% of the total time.

OL test

OL was used to evaluate short-term spatial recognition memory in mice (57). In this task, mice are presented to two identical objects in a training phase. In the test session, one of the objects is moved to a different position inside the arena. Mice with preserved memory are expected to spend more time exploring the displaced object than the object in the original position. OL was carried out in an open field arena measuring 30 × 30 × 45 cm. Initially, animals were subjected to a habituation session of 30 min. Twenty-four hours later, animals were exposed to two identical objects side-by-side for 5 min (training session). Two hours later, animals were exposed to the same pair of objects for 5 min, but one of the objects had been relocated to the opposite corner of the arena (test session). The time spent exploring each object in both sessions was recorded. The results were expressed as the percentage of time spent exploring each object during the test session and analyzed using one-sample Student’s t test, comparing the mean exploration time for each object with the chance value of 50%. Animals that recognize the relocated object as such explore it for more than 50% of the total time.

Statistics

Statistical analysis was carried out using Student’s t test when two different experimental conditions were compared or by one-way ANOVA followed by appropriate post-hoc test when more than two conditions were compared.

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